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Biochem. J. (1960) **77**, 209

Fractionation of C-Esterase from the Hog's Kidney Extract

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(*Received* 9 *February* 1960)

Evidence has been adduced for the presence of a new type of esterase in hog's kidney extract, in addition to B-esterase and an enzyme (DFPase) hydrolysing di*iso*propyl phosphorofluoridate (Bergmann, Segal & Rimon, 1957; Bergmann & Rimon, 1958). This so-called 'C-esterase' was characterized by the following properties: (*a*) It is not inhibited by di*iso*propyl phosphorofluoridate and related organic phosphates, in contrast with B-esterase (Aldridge, 1953). (*b*) It does not hydrolyse these phosphates, in contrast with A-esterase (Aldridge, 1953). (*c*) It is activated by concentrations of *p*-chloromercuribenzoate that fully inhibit DFPase (Bergmann *et al.* 1957).

Difficulties were, however, encountered in the complete characterization of the new enzyme because it could not be separated from the other hydrolytic enzymes present and because dialysis led to a partial loss of activity in some preparations whereas others, which had passed through exactly the same purification process, were insensitive to dialysis (Bergmann & Rimon, 1958).

Although the observations reported left little doubt about the definition of C-esterase as a new enzyme, final proof of its existence as an individual entity, and likewise clarification of its peculiar properties, awaited its separation from the other hydrolytic enzymes present in kidney extract. This has now been achieved by the use of diethylaminoethylcellulose as adsorbent.

MATERIALS AND METHODS

Enzyme preparations. Crude C-esterase was prepared as described by Bergmann *et al.* (1957), but the ethanol precipitate was dissolved in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) succinate buffer, pH 8·0, for passage through the diethylaminoethyl (DEAE)-cellulose column.

Chymotrypsin was a crystalline product of Worthington Biochemical Corp., Freehold, N.J., U.S.A. The activity was 2000 μmoles/ml./hr./mg. of protein, when the hydrolysis of 25 mM-phenylalanine ethyl ester (Adkins & Billica, 1948) was measured by the manometric method of Park & Plaut (1953).

Enzymic activities. Activity of DFPase was measured at pH 7·4 against 5 mM-di*iso*propyl phosphorofluoridate (DFP) by the manometric method.

C-Esterase was determined at 28°, after incubation with 0·01 M-tetraethyl pyrophosphate for 5 min. (Bergmann & Rimon, 1958). Hydrolysis of the substrate, mM-*p*-nitrophenyl acetate, was measured at pH 7·6 by the spectrophotometric method described by Bergmann, Rimon & Segal (1958). In some experiments, 0·4 M-diacetin served as substrate, hydrolysis being measured by the manometric method.

B-Esterase activity was obtained as the difference between the total enzymic hydrolysis of *p*-nitrophenyl acetate minus the rate due to the catalytic effect of C-esterase.

For the experiments with chymotrypsin, both 0·4 mM-*p*-nitrophenyl acetate and mM-*p*-methoxyphenyl acetate served as substrates, hydrolysis being measured by the spectrophotometric method at 28°.

Buffers. For the manometric method, composition of buffer used was: 0·1 M-NaCl; 25 mM-$NaHCO_3$; 0·04 M-$MgCl_2$; 0·1% of gelatin. The gas phase consisted of air + CO_2 (95:5, v/v). The pH of all solutions was adjusted to 7·4 before the experiment, which was carried out at 37°.

For both B- and C-esterase, 5 mM-tris buffer, pH 7·6, was used, when the hydrolysis of phenyl acetates was measured.

For the experiments on pH-dependence the following buffers covered the range required: 0·1 M-sodium acetate for pH 5·0–6·5; 0·1 M-sodium phosphate for pH 6·5–8; 0·1 M-sodium pyrophosphate for pH 8–9·5; 0·1 M-sodium borate for values above pH 9·5.

Inhibitors. Sodium *p*-chloromercuribenzoate was a commercial product of Sigma Chemical Co., St Louis, Mo., U.S.A. Mercaptoethanol and β-mercaptopropionic acid were Eastman Kodak Co. products and were redistilled before use. Tetraethyl pyrophosphate was a gift of Dr A. D. Toy, Victor Chemical Works, Chicago, Ill., U.S.A.

Protein analysis. Protein was determined with Folin's reagent according to the procedure of Herriot (1942).

Column chromatography. DEAE-cellulose (Eimer and Amend, New York, N.Y., U.S.A.) was washed first with 0·1 N-NaOH and then with water until the effluent gave a neutral reaction. Subsequently the washing was continued with 0·1 N-HCl and water, and the adsorbent was finally equilibrated with tris buffer, pH 8·0, for 24 hr. The properties of this material change upon repeated use, although between two loadings the whole purification procedure was again applied. The results reported refer to the second and third runs. After the fourth run it was necessary to discard the adsorbent.

The column used had the following dimensions: length, 13 cm.; diameter, 13 mm.; wt. of adsorbent, 3·5 g.

RESULTS

Separation of C-esterase from B-esterase and DFPase. When a solution of crude C-esterase in tris buffer was passed through the DEAE column,

partial separation of the three hydrolytic enzymes was effected (Fig. 1). C-Esterase is the least adsorbable component and appears in two main fractions: (1) as a protein, which is not held at all by the column (the second peak of this enzyme is already mixed with that of B-esterase); (2) in the fraction obtained by elution with buffer + 0·02 M-NaCl. In fractions no. 23–25, only a small activity of C-esterase type was observed. The properties of these higher fractions are discussed below.

The fact that all three hydrolases in hog's kidney extract give at least two peaks may indicate that these enzymes are not homogeneous proteins.

The separation procedure was repeated many times with exactly the same results, thus providing final proof that C-esterase represents an independent enzymic entity. The high degree of purity of fraction 4, for example, is indicated by its specific activity (1·4 μmoles of *p*-nitrophenyl acetate/ml./hr./mg. of protein). For reasons which will become clear in the following, this preparation will be called henceforth 'type I C-esterase'. Undoubtedly it still contains inactive protein as contaminant.

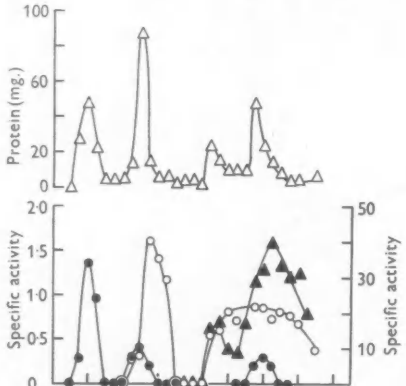

Fig. 1. Separation of the hydrolases of hog's kidney extract by fractionation on a DEAE-cellulose column. In this experiment 16 ml. of a solution containing 31 mg. of protein/ml. in tris buffer, pH 8, and exhibiting the following enzymic activities was used: B-esterase, 0·67 μmole of *p*-nitrophenyl acetate/ml./hr./mg. of protein; C-esterase, 0·48 μmole of *p*-nitrophenyl acetate/ml./hr./mg. of protein; DFPase, 17·0 μmoles of di*iso*propyl phosphorofluoridate/ml./hr./mg. of protein. All fractions represent 10 ml. ○, B-esterase (left ordinate); ●, C-esterase (left ordinate); ▲, DFPase (right ordinate); △, protein (mg.) for total volume of each fraction. Graded elution: fraction nos. 1–7, tris buffer, pH 8·0; 8–12, tris buffer + 0·02 M-NaCl, pH 8·0; 13–15, tris buffer + 0·04 M-NaCl, pH 8·0; 16–20, tris buffer + 0·08 M-NaCl, pH 8·0; 21–25, tris buffer + 0·16 M-NaCl, pH 8·0; 26–32, tris buffer + 0·32 M-NaCl, pH 8·0.

The purified enzyme is stable over long periods and can be freeze-dried without loss of activity. Since the colour of the crude kidney extract is retained in the upper part of the column, the freeze-dried form is almost colourless. It is now possible to prepare concentrated solutions with high hydrolytic power for use with the spectrophotometric method.

Properties of type I C-esterase. The availability of C-esterase free of other hydrolases made it possible to study in detail some of the properties that previously were not understood. Crude extracts, containing a 'dialysable cofactor' (Bergmann *et al.* 1957), always lost the latter during passage through the column (Table 1). Activation by sodium *p*-chloromercuribenzoate, the most curious feature of all crude enzyme preparations, was completely missing in type I C-esterase (Table 2). This observation suggested that on the column a component, which acted as 'native' inhibitor, had been removed. The latter probably is effective by virtue

Table 1. *Influence of dialysis on the activity of crude C-esterase of type I and of type II*

Fraction no. 24 was incubated with 0·2 mM-*p*-chloromercuribenzoate (CMB) for 10 min. at room temperature. Dialysis was carried out during 48 hr. against 5 mM-tris buffer, pH 8·0. Thereafter 0·01 M-tetraethyl pyrophosphate was added. After incubation for 5 min., activity was measured at pH 7·6, with mM-*p*-nitrophenyl acetate as substrate, by the spectrophotometric method.

	Activity (μmoles/ml./hr.)	
	Before dialysis	After dialysis
Crude C-esterase	15·0	8·5
Type I C-esterase (fraction no. 4)	6·8	6·8
Type II C-esterase after activation by CMB (fraction no. 24)	41·0	41·0

Table 2. *Effect of* p*-chloromercuribenzoate on the activity of crude and type I C-esterase*

The enzyme was incubated with 0·01 M-tetraethyl pyrophosphate for 5 min. and then with *p*-chloromercuribenzoate for 10 min. Activity was measured as described in Table 1.

	Rate of hydrolysis (μmoles/ml./hr.)		
		After incubation with	
	Without treatment with CMB	0·2 mM-CMB	0·02 mM-CMB
Crude C-esterase	15·0	—	42·0
Type I C-esterase	6·8	6·8	6·8

Table 3. *Effect of glutathione on crude C-esterase before and after activation with* p-*chloromercuribenzoate*

The enzyme was first incubated with 0·01 M-tetraethyl pyrophosphate for 5 min. and then with 0·08 mM *p*-chloromercuribenzoate (CMB) for 30 min. It was then treated with 0·03 M-glutathione for 30 min. This preparation was compared with an enzyme that had undergone the same treatment with the exception of treatment with CMB. Activity was measured against 0·4 M-diacetin by the manometric method.

	Rate of hydrolysis (μmoles/ml./hr.)
Crude C-esterase	84
Crude C-esterase + glutathione	71
Crude C-esterase + CMB	494
Crude C-esterase + CMB + glutathione	71

of an SH group, which becomes bound to the mercurial compound. Such an assumption is in accordance with previous observations on crude C-esterase that activation by *p*-chloromercuribenzoate can be reversed, wholly or partially, by glutathione, whereas the latter inhibits slightly (see Table 3).

Inhibitory action of other fractions from the column on type I C-esterase. It appeared possible that the native inhibitor was separated by adsorption on the column. Therefore a number of experiments was carried out to detect the inhibitor in the peak fractions of B-esterase and DFPase. However, addition of fractions nos. 12, 20 and 27, which after treatment with tetraethyl pyrophosphate had no catalytic effect on the hydrolysis of *p*-nitrophenyl acetate, to fraction no. 4 did not alter the effect of the latter on ester hydrolysis.

This result can be explained in two ways: either the native inhibitor was missing from all fractions examined (e.g. it may not be possible to elute the inhibitor from the column even by concentrated NaCl solutions) or it does not have an effect on pure type I C-esterase, in contrast with its influence on the crude enzyme.

Effect of sulphydryl compounds on type I C-esterase. In view of the above-mentioned results, the effect of sulphydryl compounds on the purified type I C-esterase was studied. Both mercaptoethanol and β-mercaptopropionic acid were entirely inactive. The properties of this enzyme were thus fundamentally different from those of crude C-esterase, which is strongly inhibited by both sulphydryl derivatives (Fig. 2).

Properties of C-esterase from higher fractions. Different results, however, were obtained with the C-esterase present in fractions nos. 23–25. Here the above-mentioned two mercapto derivatives were strongly inhibitory (Table 4). In addition, *p*-chloromercuribenzoate produced activation of

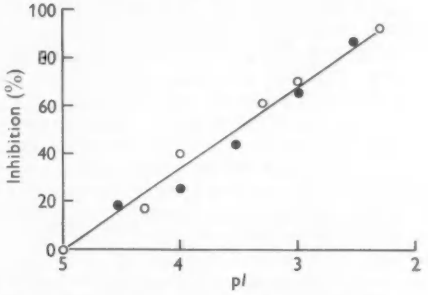

Fig. 2. p*I*–activity curve for the system crude C-esterase–sulphydryl compounds. Crude C-esterase was incubated at room temperature with varying concentrations of mercaptoethanol (●) or β-mercaptopropionic acid (○) for 15 min. Activity was measured against 0·4 M-diacetin by the manometric method. p*I* = − log concentration of inhibitor.

Table 4. *Inhibition of the two types of free C-esterase by sulphydryl compounds*

Fraction no. 24 was incubated for 5 min. with 0·01 M-tetraethyl pyrophosphate. Inhibition with mM concentrations of the mercapto derivatives was carried out for 10 min. and activity determined as described in Table 1.

	Rate of hydrolysis (μmoles/ml./hr.) by C-esterase	
Inhibitor added	Type I (fraction no. 4)	Type II (fraction no. 24)
None	6·8	1·3
Mercaptoethanol	6·8	0·7
β-Mercaptopropionic acid	6·8	0·65

Table 5. *Unmasking of 'hidden' C-esterase by* p-*chloromercuribenzoate*

B-Esterase activity was eliminated by treatment with 0·01 M-tetraethyl pyrophosphate for 5 min. *p*-Chloromercuribenzoate (CMB; 0·2 mM) was added to each fraction and the mixture was incubated at room temperature for 10 min. Thereafter activity was measured as described in Table 1. As control, the fractions untreated with CMB were used.

	C-Esterase activity (μmoles/ml./hr.)	
Fraction no.	Before activation with CMB	After activation with CMB
21	0	0
22	0	2·7
23	1	41·0
24	1·3	41·0
25	0·4	11·4
27	0	4·5
28	0	12·0
29	0	2·8
30	0	1·0
31	0	0

a much higher degree than that observed with the crude enzyme. This aroused the suspicion that these fractions contained, in addition to the small quantity of free C-esterase, a much larger proportion of hidden enzyme, i.e. enzyme bound to a native inhibitor. This assumption was confirmed by a systematic study of activation by *p*-chloromercuribenzoate, which revealed that, even in fractions completely devoid of C-esterase activity, the latter could be unmasked by treatment with the organic mercurial compound (Table 5). The enzyme in fractions nos. 22–30, which we designate as 'type II C-esterase', appears again concentrated in two peaks, like all other hydrolases in the kidney extract used.

These experiments led to the conclusion that most or all of type II C-esterase in the higher fractions was bound to a native inhibitor, the latter acting by virtue of an SH group. A search for this inhibitor in the free state was carried out among the higher fractions devoid of free C-esterase. However, fractions nos. 26–30 had no inhibitory effect on the free C-esterase in fractions nos. 23–25. Thus, in those fractions which contain neither free inhibitor nor free type II C-esterase, a stoicheiometric enzyme–inhibitor complex must be present.

Non-identity of the native inhibitor with DFPase. The above-mentioned results support the assumption of an SH group as the essential part of the native inhibitor. It was shown by Mazur (1946) that DFPase possesses also a sulphydryl group. This fact, combined with the observation that hidden C-esterase is present only in fractions exhibiting great DFPase activity, suggested that the latter enzyme may actually represent the native inhibitor of type II C-esterase. Since there is no free, i.e. excess of, inhibitor present in fractions nos. 22–30, the stoicheiometric complex C-esterase–native inhibitor would have to be identical with a complex C-esterase–DFPase, which includes all of the DFPase available. This means that DFPase, when entering the complex, retains its enzymic activity whereas C-esterase does not. Such a possibility cannot be excluded. However, the following considerations contradict the assumption that DFPase represents the native inhibitor.

If DFPase participates as one component in the stoicheiometric enzyme–inhibitor complex, then the ratio

$$\frac{\text{C-esterase recoverable by } p\text{-chloromercuribenzoate}}{\text{original DFPase}}$$

should be constant, since there is no excess of inhibitor available in any of the fractions tested. However, the data in Table 6 give evidence to the contrary. Therefore the native inhibitor is a separate, as yet unidentified, entity.

Further characterization of type II C-esterase. The fact that the hidden C-esterase appears only in higher fractions could be due to either of two factors: (i) this enzyme may be structurally similar to type I C-esterase, but by combination with the native inhibitor it is altered in such a way as to exhibit increased adsorbability on DEAE-cellulose and to require a high concentration of NaCl for elution; (ii) the structure of type II C-esterase itself may be essentially different. A decision between these two alternatives can be reached by rechromatography of the enzyme activated by *p*-chloromercuribenzoate on a DEAE-cellulose column. Pertinent experiments showed that the hidden enzyme, after its liberation from the native inhibitor, again is firmly adsorbed and requires 0·16 M-NaCl for its elution, like the original enzyme–inhibitor complex. Therefore the behaviour of type II C-esterase is characteristic for the enzyme

Table 6. *Evidence for the non-identity of DFPase with the 'native inhibitor' of C-esterase*

In each fraction DFPase activity was measured as described in the Materials and Methods section. C-Esterase activity was determined both before and after activation with *p*-chloromercuribenzoate (CMB) (see Table 5). The difference between C-esterase after and before CMB treatment was used as a measure of hidden C-esterase activity, to calculate the ratio shown in the second column.

Fraction no.	Hidden C-esterase / DFPase
22	0·22
23	0·35
24	0·74
25	0·54
27	0·59

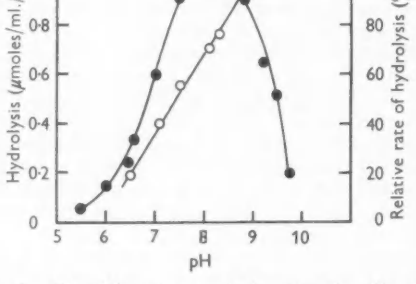

Fig. 3. pH–activity curves of chymotrypsin. For the hydrolysis of 0·4 mM-*p*-nitrophenylacetate (○, left ordinate) 1 mg. of crystalline chymotrypsin/ml., and for *p*-methoxyphenyl acetate (●, right ordinate), 0·5 mg. of crystalline chymotrypsin/ml., was used.

itself and independent of the presence or absence of the native inhibitor.

Finally, type II C-esterase, after activation by *p*-chloromercuribenzoate, does not suffer any loss of activity during dialysis, a property shared with the type I enzyme, but contrasting with the behaviour of the crude enzyme used in the present investigation (Table 1).

Effect of pH *on the hydrolytic activities of either type of C-esterase.* In view of the isolation of two different types of C-esterase from hog's kidney extract, it was of interest to determine their pH–activity curves. Both forms of the enzyme revealed again a curious property, observed previously with the crude C-esterase (Bergmann & Rimon, 1958). The pH–activity curve for *p*-methoxyphenyl acetate is bell-shaped, whereas the hydrolysis of *p*-nitrophenyl acetate accelerates steadily with increasing pH. This behaviour is not unique for C-esterases. We have found that chymotrypsin exhibits similar pH–activity curves for the above-mentioned two substrates (Fig. 3). The effect of pH on various hydrolytic reactions catalysed by C-esterases apparently is independent of the degree of purity of the enzyme or the presence or absence of a mixture of different types.

DISCUSSION

The present experiments establish beyond doubt the existence of C-esterase as a separate enzyme. In addition they reveal that in hog's kidney extract two different types of this esterase are present, the properties of which are summarized in Table 7.

These observations provide a rational explanation for the varying degrees of activation of crude C-esterase by *p*-chloromercuribenzoate (between 50 and 800 %). The crude preparation contains not only a mixture of type I and II esterase in various proportions, but the latter may be masked to different extents by bonding to the native inhibitor.

Since the discovery of C-esterase, many unsuccessful attempts were made to purify this enzyme, i.e. to separate it from the accompanying hydrolases. Neither the use of various salts nor the use of organic solvents effected a separation, although the ratio between DFPase, B-esterase and C-esterase differed from one fraction to the other. It is now apparent that the crude C-esterase contains at least four components, differing in their structure, as indicated by the double peak for type I (Fig. 1) and type II (Table 5) C-esterase. Only the much more delicate selectivity of DEAE-cellulose makes this separation possible. However, there is no evidence that the peak fractions isolated in the present investigation represent already homogeneous enzyme proteins.

Table 7 and likewise the pH–activity curves demonstrate a close similarity between the two types of C-esterase. This raises the question whether type I may be derived from type II by partial degradation.

Inhibition by sulphydryl compounds is a common phenomenon among enzymes bearing a metal atom in the active centre. However, no information on this point will be available for type II C-esterase until this enzyme can be obtained free of the other enzymes and of its native inhibitor. In this connexion it should be recalled that, in a large percentage of our crude enzyme preparations, C-esterase lost part (40–50 %) of its activity during dialysis. However, this was no longer the case with the enzymes recovered from the DEAE-cellulose column (type II being dialysed after activation by *p*-chloromercuribenzoate). Therefore inhibition by sulphydryl compounds is in no way connected with the presence of the dialysable component of C-esterase.

The similarity of the pH–activity curves of C-esterase and chymotrypsin, when phenyl acetates serve as substrates, indicates that the hydrolytic mechanisms of these enzymes cannot be fundamentally different. In view of the evidence for the presence of imidazol in the active centre of chymotrypsin (Dixon & Neurath, 1957), it may be assumed that this group also represents the essential component in the active surface of C-esterases.

Thus there exist two groups of esterases in animal tissues, both of which make use of imidazol for their catalytic mechanism: one group that requires, in addition, serine and therefore is

Table 7. *Survey of the characteristics of the two types of C-esterase*

	Type I C-esterase	Type II C-esterase
Inhibition by organophosphorus compounds	Nil	Nil
Hydrolysis of organophosphorus compounds	Nil	Nil
Inhibition by sulphydryl compounds	Nil	Strong
Adsorption on DEAE-cellulose at pH 8·0	Nil	Strong
Dialysis	No effect	No effect
Native appearance	Free	Predominantly in combination with native inhibitor

sensitive to phosphate inhibition (B-esterases); a second group which supposedly works without serine (A- and C-esterases), although no positive evidence is available on this point.

SUMMARY

1. C-Esterase was separated from other hydrolytic enzymes in hog's kidney extract by fractionation on a diethylaminoethylcellulose column. The non-adsorbable protein contained type I C-esterase, free of B-esterase and an enzyme (DFPase) hydrolysing di*iso*propyl phosphorofluoridate.

2. In addition, most of the fractions with DFPase activity contained a second type of C-esterase, predominantly in hidden form, i.e. linked to a native inhibitor.

3. Type II C-esterase is liberated from this inhibitor by treatment with *p*-chloromercuribenzoate. It differs from type I C-esterase by its sensitivity towards sulphydryl inhibitors and by its stronger adsorbability on diethylaminoethylcellulose.

4. Both C-esterases show an abnormal pH–activity curve towards *p*-nitrophenyl acetate. With increasing pH the hydrolytic rate rises steadily. This property is also shared by chymotrypsin.

5. The properties of the two types of purified C-esterase can explain most of the observations made on crude enzyme mixtures.

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Biochem. J. (1960) **77**, 214

The Partial Purification and Properties of Thyroxine Dehalogenase

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In most mammals, dehalogenation is one of the most important metabolic transformations of L-thyroxine, 3:5:3′-tri-iodo-L-thyronine and related substances (Pitt-Rivers & Tata, 1959). The physiological significance of dehalogenation of thyroid hormones remains doubtful although numerous reports have appeared in recent years on the deiodination of thyroid hormones by incubation with slices, homogenates or extracts of various tissues (Albright, Larson & Tust, 1954; Larson, Tomita & Albright, 1955; Sprott & Maclagan, 1955; Béraud & Vannotti, 1956; Tata, Rall & Rawson, 1957; Tata, 1957, 1958*b*; Maclagan & Reid, 1957; Plaskett, 1958; Albright & Larson, 1959; Yamazaki & Slingerland, 1959; Etling & Barker, 1959*a*; Lissitzky, Bénévent, Roques & Roche, 1959). Unfortunately there is little agreement on the nature and products of enzymic deiodination. The major causes of this confusion are: (*a*) the predominant interest of some investigators in demonstrating partial deiodination of thyroxine to triiodothyronine, (*b*) variability in techniques of detection and measurement of deiodination, (*c*) failure to realize the influence of tissue source and method of enzyme preparation used and (*d*) neglect of non-enzymic processes which can occur simultaneously (Lissitzky, Michel, Roche & Roques, 1956; Tata, 1959*a*; Etling & Barker, 1959*b*; Lein & Michel, 1959).

A series of preliminary investigations were undertaken by the author to overcome the above objections before studying the enzyme system(s) involved in deiodination of the thyroid hormones. These investigations have shown that direct chromatographic analysis with multiple solvent systems is the most reliable method for measurement of deiodination (Tata *et al.* 1957; Tata, 1957, 1958*b*); direct chromatography has also been used by others (Lissitzky, Bénévent, Roques & Roche, 1959; Yamazaki & Slingerland, 1959; Etling & Barker, 1959*a*). It is, also, now possible to distinguish between true enzymic dehalogenation and one

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type of non-enzymic process, the phenomenon of 'instability' (Tata, 1959*b*, 1960*a*). Although liver and kidney deiodinate thyroxine and tri-iodothyronine more actively than any other tissue, both *in vivo* and *in vitro* (Pitt-Rivers & Tata, 1959), skeletal muscle was selected for studies on the dehalogenating enzyme. It was shown that with skeletal muscle, whether as slices, homogenate or soluble extract, deiodination of thyroid hormones was the only reaction involving these hormones that was catalysed (Tata, 1957, 1958*b*). With liver and kidney, complicating side-reactions occur, leading to the formation of metabolites which are themselves substrates for the dehalogenase. With kidney tissue, moreover, the dehalogenating activity is abolished by the loss of tissue integrity (Tomita, Lardy, Larson & Albright, 1957), whereas a 'soluble' preparation of dehalogenase can be obtained from skeletal muscle (Tata, 1958*b*).

The dehalogenating enzyme present in the thyroid gland, first described by Roche and his colleagues (Roche, Michel, Michel & Lissitzky, 1952; Roche, Michel, Michel, Gorbman & Lissitzky, 1953), has high affinity for the iodotyrosines but does not attack thyroxine or tri-iodothyronine. It has been further characterized by Stanbury (1957) and Stanbury & Morris (1958) as a reduced triphosphopyridine nucleotide-dependent enzyme present in the microsomal fractions of thyroid as well as liver tissues. It has been recently shown that the iodotyrosine deiodinase is different from the enzyme in muscle, which is not reduced triphosphopyridine nucleotide-dependent but is activated by Fe^{2+} ions and flavin derivatives (Tata, 1959*c*).

The purpose of this paper is to present a method for the partial purification of the enzyme present in rabbit skeletal muscle and to describe its important properties. It will be shown that thyroxine is the substrate for which the enzyme has the highest affinity and that, besides deiodination, it can also catalyse debromination of brominated analogues. The term 'thyroxine dehalogenase' is therefore proposed for this enzyme.

EXPERIMENTAL

Substrates. All enzyme incubations were performed with radioactive substrates labelled in the *ortho* position to the phenolic OH group. The following ^{131}I-labelled compounds were either obtained from Abbott Laboratories, Oak Ridge, Tenn., U.S.A., or prepared by radio-iodination of the corresponding 3:5-di-iodothyronine derivative or of tyrosine: L-thyroxine (L-T_4), D-thyroxine (D-T_4), DL-thyroxine, 3:5:3′-tri-iodo-L-thyronine (T_3), 3:5:3′:5′-tetraiodothyroacetic acid, 3:5:3′-tri-iodothyroacetic acid, 3-iodo-L-tyrosine (MIT) and 3:5-di-iodo-L-tyroxine (DIT). The specific radioactivity of the iodothyronine derivatives was 15–24 mc/μmole (Abbott Laboratories) or 4–7 mc/μmole (this laboratory) and that of the iodotyrosines 5–9 mc/μmole. Radioactive 3-bromo-L-tyrosine, 3:5-dibromo-L-tyrosine, 3:5:3′:5′-tetrabromo-DL-thyronine and 3:5:3′-tribromo-DL-thyronine were prepared by the radiobromination (^{82}Br) of L-tyrosine or 3:5-dibromo-DL-thyronine (3·5 g. atoms of Br/mole of amino acid) to give a specific radioactivity of 0·8–1·4 mc/μmole. The mono- and di-substituted radioactive compounds were separated by preparative paper chromatography. ^{131}I and ^{82}Br were obtained from A.E.R.E., Harwell. The unlabelled compounds were the products of Glaxo Laboratories, Warner-Chilcott Laboratories or synthesized by Dr R. Pitt-Rivers, F.R.S. Stock solutions, at suitable concentrations of all compounds, were prepared in 10% (v/v) propane-1:2-diol in water.

Buffers. All buffer solutions were prepared according to Gomori (1955) in demineralized water. The buffer used for tissue extraction was 2-amino-2-hydroxymethylpropane-1:3-diol (tris)–HCl–KCl, pH 7·1, I 0·22 (I was 0·10 and 0·12 with respect to tris–HCl buffer and KCl respectively).

Enzyme preparations. All procedures, unless otherwise stated, were performed at 0–2°.

(*a*) Crude extract. Muscle was removed from the leg, thigh and back of 8–12-month-old rabbits killed by a blow on the neck. Care was taken to avoid contamination of tissue fragments with blood. Tissue (550 g.) was suspended in 2 l. of tris–HCl–KCl buffer, pH 7·1, I 0·22, and finely chopped with scissors. The suspension was homogenized in a MSE Ato-Mix blender and the homogenate centrifuged at 20 000 *g* for 20 min.; the sediment was discarded.

(*b*) Decrease in ionic strength. The crude supernatant was dialysed against 20 vol. of tris–HCl–KCl buffers of increasing dilutions until a value for I of 0·015 was reached. The precipitate formed was discarded by centrifuging at 7000 *g* for 10 min., leaving a slightly turbid supernatant.

(*c*) Heat treatment. The extract of I 0·015 was brought to 45° with stirring and maintained at that temperature ±1° for 5 min., when a copious precipitate was formed. The suspension was chilled in ice and centrifuged at 7000 *g* for 10 min. to yield a clear supernatant that was enzymically active.

(*d*) First ammonium sulphate precipitation. To the above-mentioned solution sufficient saturated $(NH_4)_2SO_4$, adjusted to pH 8·0 with NH_3, was slowly added to 48% saturation. The suspension was left to stand overnight before collecting the active precipitate by centrifuging. The precipitate was suspended in 120–150 ml. of tris–HCl–KCl buffer, pH 7·1, I 0·20, stirred vigorously and dialysed first against 100 vol. of diluted buffer (I 0·015) and then against running water for 18 hr. The insoluble material was removed by centrifuging at 20 000 *g* for 15 min.

(*e*) Adsorption on calcium phosphate gel. One-year-old calcium phosphate gel (19 mg./ml.), prepared according to Keilin & Hartree (1938) (50 ml.), was stirred into 100 ml. of the supernatant from the last step. After 30 min. the suspension was centrifuged, the supernatant discarded and the precipitate was washed and centrifuged three times with 30 ml. of 0·15 M-phosphate buffer, pH 7·0. The washings were discarded and the precipitate was stirred into 30 ml. of 0·55 M-phosphate buffer, pH 7·0, before being centrifuged at 5000 *g* for 10 min. This was repeated twice, the three washings were pooled and dialysed against running water for 18 hr.

(*f*) Second ammonium sulphate precipitation. To 120 ml. of the above-mentioned dialysed solution was added sufficient saturated $(NH_4)_2SO_4$ solution, pH 8·0, to give 42 % saturation. After 15 hr. the precipitate was recovered by centrifuging, suspended in tris–HCl–KCl buffer (*I* 0·10) and dialysed first against running water for 12 hr. and then for 10 hr. against demineralized water. The slightly opalescent solution was clarified by centrifuging at 20 000 *g* for 20 min. The resulting supernatant represented the purified thyroxine dehalogenase used for most studies of enzyme properties reported below.

Storage of enzyme. The active preparation was usually stored in solution in water or 0·05 M-tris–HCl buffer, pH 7·1, at 0–2° without freezing. The concentration was adjusted to 10–15 mg. of protein/ml. In some cases the enzyme was stored as a freeze-dried preparation, although this procedure caused some loss of enzyme activity.

Enzyme assay. The enzyme was incubated aerobically at 37°, with shaking, in a 25 ml. conical flask, the final volume of contents being 2·5–3·0 ml. The substrate in 10 % propane-1:2-diol solution was added last; the concentration of the substrate was so arranged that the final concentration of the organic solvent would not exceed 0·02 % (v/v) in the presence of the enzyme. The course of enzymic dehalogenation was followed by chromatographic analysis of samples withdrawn periodically during incubation (Tata *et al.* 1957). The distribution of radioactivity in different fractions on chromatograms was automatically recorded and calculated by an integration device or by planimetry of zones of radioactivity as follows:

$$\mu\text{m-moles of } T_4 \text{ dehalogenated} = \frac{\text{area under } T_4 \times \mu\text{m-moles of } T_4 \text{ added}}{\text{area under all peaks}}.$$

Corrections were made for the amount of contaminating radioactive iodide or bromide originally present.

It was assumed that substrate was absent, or present in negligible amounts only, in the enzyme preparation and that isotopic exchange did not occur. One enzyme unit was tentatively defined as the amount of enzyme necessary to catalyse the dehalogenation of 1 μm-mole of L-thyroxine/10 min.

Enzymic dehalogenation was distinguished from an apparently similar but spontaneous reaction (Tata, 1959*a*) by a test based on the phenomenon of 'stabilization' effect of thyroxine-binding proteins (Tata, 1959*b*, 1960*a*). This test was performed by the addition of 25–30 mg. of dried whole human-blood plasma to the incubation mixture 1 min. before the last sample was withdrawn for chromatography. Corrections were applied for the fraction of radioactivity which 'reappeared' in the substrate fraction. The addition of plasma proteins, with thyroxine, also served to stop the enzyme reaction.

Chromatography. Ascending chromatography on Whatman no. 1 paper was used for enzyme assay and identification of the products formed. Whatman no. 3 paper was used for preparation of labelled compounds. Two solvent systems were used: (*a*) butan-1-ol–acetic acid–water (78:10:12, by vol.); (*b*) butan-1-ol–dioxan (80:20,v/v), previously shaken with aq. 2 N-NH_3 solution, was used to detect the possible formation of tri-iodothyronine from thyroxine. The short half-life (35·9 hr.) of ^{82}Br necessitated the acceleration of paper chromatography by centrifugal force for ^{82}Br-labelled compounds. An apparatus recently developed in this laboratory was used for this purpose according to techniques described elsewhere (Tata & Hemmings, 1960).

Electrophoresis. The analysis of tissue proteins or serum proteins was performed by horizontal-paper electrophoresis in veronal–acetate–KCl buffer, pH 7·4, *I* 0·1 (Kotter, Lenz & Prändl, 1955), or 0·05 M-veronal buffer, pH 8·6 (Block, Durrum & Zweig, 1955). The purification of enzyme was also followed by starch-gel electrophoresis in borate buffer, pH 8·8, according to Smithies (1955). Paper electrophoresis was used for studying protein-binding of thyroxine according to methods described by Robbins & Rall (1957). Preparative electrophoresis on columns of cellulose (treated with acid and ethanol) was performed according to Flodin & Porath (1954) for the isolation of a cellular thyroxine-binding fraction from a crude extract of rabbit muscle.

Protein-nitrogen determinations. The protein content was measured by the determination of nitrogen in trichloroacetic acid-insoluble material according to Lang (1958).

RESULTS

Partial purification of the enzyme

The progressive enrichment of thyroxine dehalogenase in a typical procedure of purification is summarized in Table 1. It is essential to prepare the tissue homogenate in a medium of relatively high ionic strength (*I* 0·22) in order to obtain a 'soluble' enzyme extract, although in the later stages of purification the enzyme was soluble in water. The enzyme could be 'salted out' with ammonium sulphate only in a broad zone of 32–50 % saturation. For most studies reported below, preparations of 60–90-fold purification were used.

Table 1. *Purification of thyroxine dehalogenase from rabbit skeletal muscle*

All enzyme assays were performed in the presence of 10 μM-flavin mononucleotide and 6 μM-Fe^{2+} ion in tris–HCl buffer, pH 7·1, over 15 min. of incubation.

Step	Volume (ml.)	Dehalogenase (units)	Protein (mg.)	Yield (%)	Purity (units/mg. of protein)	Purification
1. Homogenate	2 250	11 000	56 000	100·0	0·19	1
2. Supernatant (*I* 0·22)	1 650	9 960	22 500	90·4	0·40	2·1
3. Decrease to *I* 0·015	1 610	9 200	13 300	83·6	0·68	3·6
4. Supernatant after heat-treatment	1 595	8 950	9 160	79·1	0·95	5·0
5. Ppt. from 48 % $(NH_4)_2SO_4$	210	6 150	2 030	55·9	2·93	15·4
6. Eluate from calcium phosphate gel	150	3 280	265	29·8	11·99	63·1
7. Ppt. from 42 % $(NH_4)_2SO_4$	80	2 950	213	26·9	13·68	72·0

Cruder preparations were used in some experiments where enrichment of enzyme was not essential.

Electrophoresis indicated that even the final enzyme preparation of Table 1 was quite heterogeneous in terms of proteins present. The 10–12 skeletal-muscle proteins present in the crude extract were especially well revealed by starch-gel electrophoresis, although only four or five zones could be detected by paper electrophoresis. The method of purification used here has, however, resulted in the removal of several (five or six) inactive proteins, as was seen from a comparison of the patterns of starch-gel electrophoresis.

Stability of the enzyme

Even under the best conditions the enzyme could not be preserved for more than 2–3 weeks without substantial loss of activity. The best preparation was obtained in a solution of 10–15 mg. of protein/ml. of 0·025 M-tris or phosphate buffers, pH 7·1, at 0–2° and without freezing. Under these conditions about 25 % of the activity was lost in 4 days and 50 % in 12 days of storage. Six alternate operations of freezing and thawing of the solution destroyed all enzymic activity. Freeze-drying of a salt-free solution resulted in the loss of 30–60 % of the activity. The addition of glutathione or cysteine and storage under nitrogen offered little advantage.

Properties of thyroxine dehalogenase

The relationship between enzyme concentration and initial velocity of deiodination of thyroxine is illustrated in Fig. 1. As explained by Tata (1960*a*), the non-linearity of the uncorrected curve with small amounts of enzyme was due to the phenomenon of 'instability' of thyroxine (Tata, 1959*a*), which was responsible for the apparently higher values of deiodination as measured by chromatography. When larger amounts of enzyme were used a 'stabilization' of thyroxine could have resulted from the presence of proteins in the enzyme preparation.

The stimulating effect of Fe^{2+} ions and flavin derivatives on thyroxine dehalogenase and its inhibition by metal-complexing agents, flavin antagonists, mercurials and SH-group poisons have already been described (Tata, 1959*c*). In Fig. 2 it is shown that the activation of the enzyme by flavin mononucleotide (FMN) and Fe^{2+} ions appears to be additive to some extent. However, as it will be shown later, there was an important difference in the action of the two factors when the products of dehalogenation were studied. Similar results were observed when FMN was replaced by flavinadenine dinucleotide (FAD) or riboflavin. No metal ions were found to replace Fe^{2+} ions.

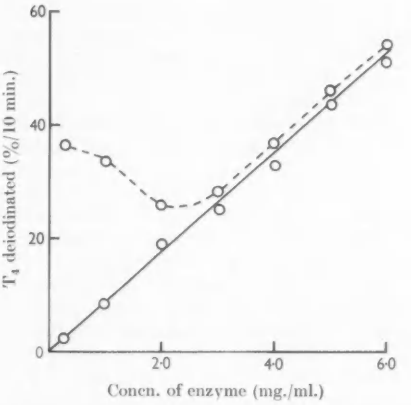


Fig. 1. Relationship between enzyme concentration and the initial rate (first 10 min.) of deiodination of L-thyroxine (5·0 μm-mole) by thyroxine dehalogenase of rabbit skeletal muscle, purification 72-fold. Each sample (2·5 ml.) was incubated in the presence of 10 μM-Fe^{2+} ion and 20 μM-flavin mononucleotide. Corrected values (—) are derived from samples to which 20 mg. of whole human plasma proteins were added 1 min. before withdrawal of a portion for chromatographic analysis. - - -, Uncorrected values.

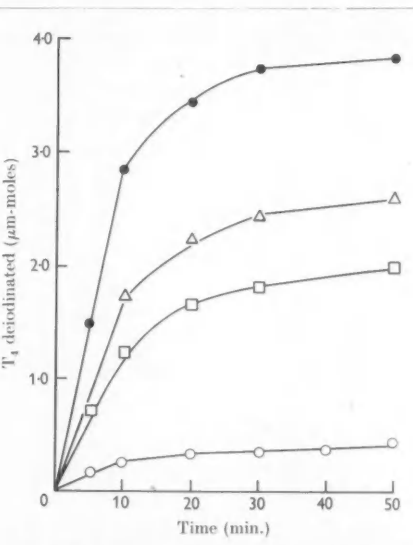


Fig. 2. Activation of thyroxine dehalogenase by Fe^{2+} ions and flavin mononucleotide (FMN). Each incubation vessel contained 12 mg. of enzyme, purification 72-fold, and 4·5 μm-moles of L-T_4 as substrate in 2·7 ml. of tris–HCl buffer, pH 7·0. Additions: ○, none; □, 0·1 μmole of FMN; △, 0·034 μg. ion of Fe^{2+}; ●, 0·1 μmole of FMN and 0·034 μg. ion of Fe^{2+}.

Among other factors affecting the enzymic reaction it was found that: (1) molecular oxygen was not necessary, (2) the dehalogenation could be catalysed under nitrogen, (3) addition of hydrogen peroxide or catalase had no effect, indicating that dehalogenation was not a peroxidative mechanism, and (4) addition of pyruvate did not modify the reaction.

The inhibition of thyroxine dehalogenase with mercuric chloride presented a peculiar pattern since the degree of inhibition was not only determined by the amount of inhibitor added but also by whether it was added before or after FMN (Table 2). There was slightly, but significantly, less inhibition if the flavin cofactor was added after Hg^{2+} ions, and the addition of increasing amounts of FMN led to some reactivation of the enzyme. The reactivation did not exceed 40–50 % of the activity in the control sample and could not be achieved by the addition of Fe^{2+} ions. No explanation is available for this phenomenon; somewhat similar behaviour has also been described by Frisell & Hellerman (1957) for kidney D-amino acid oxidase, also a flavin-dependent enzyme.

Table 2. *Influence of flavin mononucleotide on the inhibition of thyroxine dehalogenase by mercuric chloride*

The final volume was 2·7 ml.; 15 mg. of enzyme of specific activity 42, 6·0 μm-moles of L-T_4, tris–HCl buffer, pH 7·1, and 0·10 μg. ion of Fe^{2+} were added to each sample.

First addition	Amount (μmoles)	Second addition	Amount (μmoles)	Inhibition (%)
FMN	0·10	None	—	0·0
FMN	0·10	$HgCl_2$	0·01	46·5
FMN	0·10	$HgCl_2$	2·5	94·7
$HgCl_2$	2·0	None	—	96·0
$HgCl_2$	2·0	FMN	0·10	86·5
$HgCl_2$	2·0	FMN	1·0	72·3
$HgCl_2$	2·0	FMN	10·0	61·1

A study of the influence of pH of the medium on the initial velocity of reaction showed that the pH optima for thyroxine and tri-iodothyronine were not identical (Fig. 3). Similar increments of 0·8–1·0 unit in the pH optimum were observed for monohalogenated thyronine or tyrosine derivatives as compared with the dihalogenated derivatives. Fig. 3 shows also that, at nearly equimolar concentrations, thyroxine was deiodinated more rapidly than tri-iodothyronine at any pH value.

In Fig. 4 is shown the relationship between substrate concentration and initial rate of deiodination of thyroxine, tri-iodothyronine and di-iodotyrosine. The use of higher substrate concentrations was limited by the low solubility of thyroxine and related compounds. The K_m values (Michaelis constant) deduced from Fig. 4 were hence not accurate. It was, however, easier to derive a graphical value of K_m by plotting the results according to the method of Lineweaver & Burk (1934). The results of such measurements are

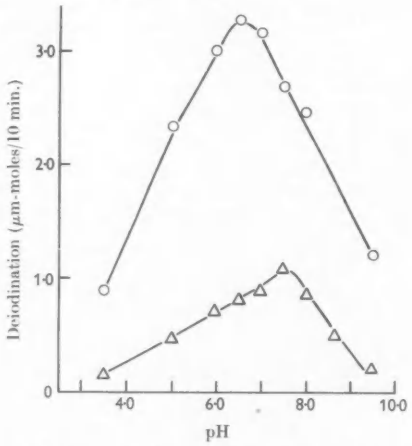


Fig. 3. Influence of pH on the initial velocity of deiodination of L-thyroxine (L-T_4) and 3:5:3′-tri-iodo-L-thyronine (L-T_3) by thyroxine dehalogenase (80-fold purification). Each vessel contained: 10 mg. of enzyme, 4·5 μm-moles of L-T_4 or L-T_3, 0·04 μg. ion of Fe^{2+} and 0·1 μmole of FMN in 2·65 ml. of buffer. ○, L-T_4; △, L-T_3. Buffers: pH 3·0–4·5, citrate; pH 4·5–5·5, acetate; pH 5·6–7·5, phosphate; pH 7·5–8·5, tris–HCl; pH 8·5–10·0, carbonate.

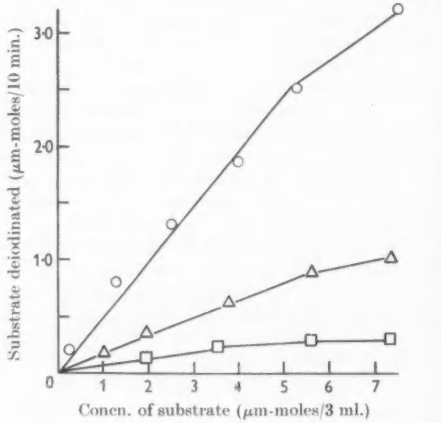


Fig. 4. Effect of substrate concentration on the initial rate of deiodination. Each vessel contained 10 mg. of enzyme (152 units), 0·2 μmole of FMN, 0·1 μg. ion of Fe^{2+} in 3·0 ml. of tris–HCl buffer, pH 7·1. Substrates: ○, L-thyroxine; △, 3:5:3′-tri-iodo-L-thyronine; □, 3:5-di-iodo-L-tyrosine.

summarized in Table 3. The presence in the enzyme preparation of a protein with strong binding affinity for any of the substrates could modify the K_m values shown in Table 3 and hence these are tentative until a pure enzyme preparation is available. However, with the same preparation, the skeletal-muscle enzyme exhibits an affinity about four times as high for thyroxine as for triiodothyronine and an affinity 15–20 times as high as for di-iodotyrosine. The enzyme was almost equally active in deiodinating L- or D-thyroxine and was remarkably active in catalysing the debromination of brominated thyronines. This is therefore one of the rare examples of enzymic debromination described.

The relative K_m values for the dehalogenase are, to a large extent, similar to the relative affinities with which serum thyroxine-binding protein binds thyroxine and related compounds (Robbins & Rall, 1957; Tata, 1960*b*). It was therefore decided to investigate the influence of thyroxine-binding protein added to the enzyme. The term 'thyroxine-binding protein' is used in this paper to denote the human-serum protein fraction which migrates in the α_1-α_2-globulin zone on paper electrophoresis. An experiment in which whole serum was added in different amounts indicated an interference with enzymic dehalogenation which was most intense for thyroxine. As seen in Fig. 5, there was a progressive inhibition of the deiodination of thyroxine which resulted in a virtually total loss of enzyme activity when the serum was present in a final six- to seven-fold dilution. In order to decide whether this inhibitory action of serum was actually due to the binding of thyroxine by thyroxine-binding protein and albumin, experiments were performed in which every sample withdrawn from the incubation vessel was submitted to analysis by both chromatography and electrophoresis. The former determined the degree of inhibition of dehalogenase and the latter revealed the fraction of radioactive

Table 3. *Apparent Michaelis constants of thyroxine dehalogenase for some iodinated and brominated analogues of* L-*thyroxine*

Conditions were identical with those in Fig. 4.

Substrate	Michaelis constant (K_m) (μM)
L-Thyroxine	1·7
D-Thyroxine	2·4
DL-Thyroxine	2·2
3:5:3′-Tri-iodo-L-thyronine	7·4
3:5:3′:5′-Tetraiodothyroacetic acid	7·9
3:5:3′-Tri-iodothyroacetic acid	13·2
3:5-Di-iodo-L-tyrosine	30
3-Iodo-L-tyrosine	50
3:5:3′:5′-Tetrabromo-DL-thyronine	9·2
3:5:3′-Tribromo-DL-thyronine	45
3:5-Dibromo-L-tyrosine	98
3-Bromo-L-tyrosine	130

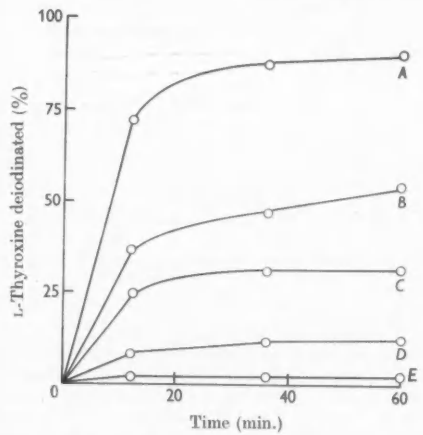


Fig. 5. Inhibitory effect of human serum on the deiodination of L-thyroxine by thyroxine dehalogenase, 61-fold purification. Each flask contained tris–HCl buffer, pH 7·2, 15 mg. of enzyme, 1·0 μm-mole of L-T_4, 0·04 μg. ion of Fe^{2+}, 0·1 μmole of FMN. Amounts of human serum: *A*, none; *B*, 0·07 ml.; *C*, 0·1 ml.; *D*, 0·275 ml.; *E*, 0·5 ml. Final volume was 2·75 ml., made up with the buffer.

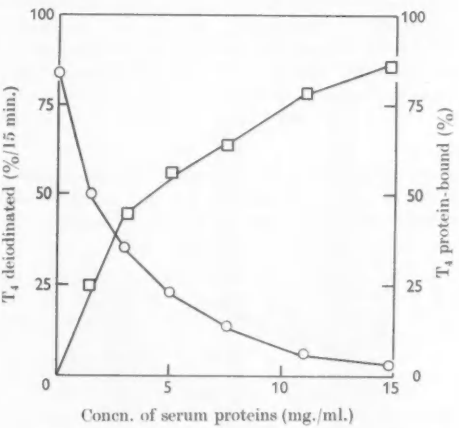


Fig. 6. Simultaneous determination of the fraction of substrate (L-thyroxine) deiodinated by thyroxine dehalogenase (○) and the fraction bound to human-serum proteins (thyroxine-binding protein and albumin) (□), when different amounts of human serum were added to the enzyme. Conditions were the same as in Fig. 5. Enzymic deiodination was determined by paper chromatography and paper electrophoresis was used for the determination of the percentage of L-thyroxine bound to serum proteins.

thyroxine bound to serum thyroxine-binding protein and albumin (it was easy to distinguish these proteins from rabbit-skeletal-muscle proteins). When the results of the two analyses were plotted, as shown in Fig. 6, it was found that for any amount of serum added the percentage inhibition of enzyme activity was nearly equal to the fraction of thyroxine bound to serum proteins. Other factors present in the serum contributed little to the inhibition. The inhibition could be reversed at any point by addition of sufficient thyroxine and it was concluded that the enzyme could only attack free or 'unbound' substrate.

Further proof of the above conclusion was provided by two more observations. (*a*) When isolated serum-protein fractions or polyvinyl pyrrolidone of different thyroxine-binding powers were added to the enzyme, the additives with higher thyroxine-binding powers were found to be the more potent inhibitors. Thus as seen in Table 4, Cohn fraction IV-4 of human plasma, an enriched thyroxine-binding fraction [thyroxine-binding protein isolated electrophoretically from Cohn fraction IV-9 (J. R. Tata, unpublished work)] and polyvinyl pyrrolidone, all known to bind thyroxine firmly (Tata, 1959*b*, 1960*a*), inhibited the enzyme at quite low concentrations. On the other hand γ-globulin, which binds thyroxine feebly, is a poor inhibitor; human-serum albumin and thyroxine-binding fraction extracted from rabbit skeletal muscle (free from dehalogenase activity and rabbit-serum thyroxine-binding protein and albumin) occupied intermediate positions. (*b*) Whole human serum, Cohn fractions IV-4 and IV-9, or isolated thyroxine-binding protein inhibited the dehalogenation of thyroxine more than that of any other iodo- or bromo-thyronine. Their effect on the dehalogenation of halogenated tyrosines was barely perceptible under similar conditions.

Table 4. *Effect of whole human serum, its protein fractions, rabbit skeletal muscle thyroxine-binding protein (C-TBP) and polyvinyl pyrrolidone on enzymic deiodination of* L-*thyroxine*

Enzyme (15 mg.) of 82-fold purification in 2·85 ml. of tris–HCl buffer, pH 7·1, with 10 μM-FMN, 5 μM-Fe^{2+} ion and 2·4 μm-moles of L-T_4. Inhibition was calculated after 15 min. of incubation.

Substance added	Amount (mg./ml.)	Inhibition of T_4 dehalogenase (%)
Whole serum	15·0*	74·5
Cohn fraction IV-4	5·0	88·0
Cohn fraction IV-9	5·0	90·5
TBP-33†	2·0	85·8
Albumin	15·0	70·0
γ-Globulin	15·0	18·6
C-TBP	10·0	57·3
Polyvinyl pyrrolidone	10·0	81·0

* Expressed as amount of serum protein in final dilution.
† Thyroxine-binding protein extracted from Cohn fraction IV-9 (unpublished work).

Products of enzymic dehalogenation

A typical pattern of distribution of ^{131}I on a chromatogram, run during the deiodination of radioactive thyroxine, is seen in Fig. 7*a*. The major product formed was iodide; however, a significant fraction of the original radioactivity of thyroxine was found in a substance of R_F 0 in different chromatographic solvent systems [Figs. 7 (*a*), (*b*)]. This material, after elution from preparative chromatograms, was found to be iodinated protein(s) present in the enzyme preparation, as shown by the release of 3-[^{131}I]iodotyrosine and a trace of 3:5-di-iodotyrosine after tryptic digestion [Fig. 7 (*c*)]. Radioactive tri-iodothyronine was found in traces in only six out of about 1500 chromatograms, as a

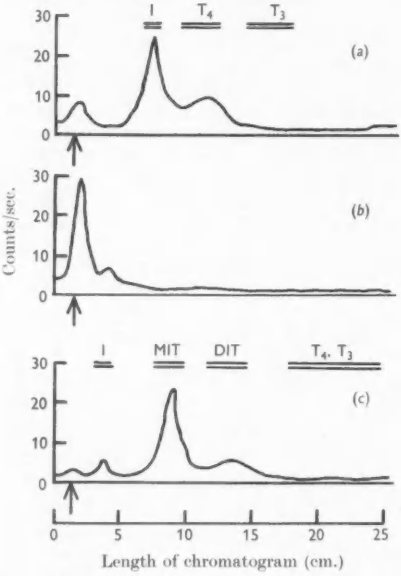


Fig. 7. Distribution of radioactivity (^{131}I) on chromatograms. (*a*) Sample withdrawn after 10 min. of enzymic deiodination of ^{131}I-labelled L-thyroxine under conditions shown in curve (○) of Fig. 2; chromatographic solvent, butan-1-ol–dioxan–NH_3. (*b*) Elution and re-chromatography of the substance of R_F 0 in chromatogram (*a*); solvent, butan-1-ol–acetic acid–water. (*c*) Chromatography of the products of tryptic hydrolysis (with crude pancreatin) of the substance of R_F 0; solvent, butan-1-ol–acetic acid–water. The position of markers is shown by solid lines above each recording. MIT, 3-iodotyrosine; DIT, 3:5-di-iodotyrosine; I, iodide. Vertical arrows indicate points of application of samples.

product of deiodination of ^{131}I-labelled thyroxine. No other major or consistent metabolite of thyroxine or other compounds was detected except for a radioactive substance of R_F 0·88–0·92 (in both acid and alkaline butan-1-ol) when labelled thyroxine was incubated in the presence of mercuric chloride. The nature of the material is not known and its formation is probably not due to enzyme action.

As seen in Fig. 8, about 20–25 % of the total ^{131}I was found in the form of iodinated protein at any time interval during the deiodination of thyroxine. It is important to distinguish between true incorporation of iodine into protein molecules as 'protein-bound ^{131}I' in these experiments from thyroxine bound to proteins in the electrophoresis experiments mentioned earlier. The formation of protein-bound ^{131}I was also observed during the deiodination of tri-iodothyronine and other iodinated compounds, but the radiobromination of proteins could not be firmly established during the debromination of brominated compounds. With the latter, the major product was the radioactive bromide.

The formation of protein-bound ^{131}I was influenced by the presence of flavin (Fig. 9). Thus although Fe^{2+} ions in the absence of FMN (or FAD) could stimulate deiodination of thyroxine more than if the flavin was added without the metal ions, there was a virtual absence of formation of protein-bound ^{131}I if the enzyme was only activated by Fe^{2+} ions. On the other hand, incubation in the presence of FMN without Fe^{2+} ions resulted in the incorporation of nearly 40 % of the radioactivity into proteins, whereas the overall dehalogenation of thyroxine proceeded at a relatively slower rate. The dependence of formation of protein-bound ^{131}I on flavin was slightly influenced by the presence or absence of light. Little ^{131}I was incorporated into protein(s) when flavin was added after most of the substrate had been deiodinated by the Fe^{2+} ion-activated enzyme.

Species and organ distribution of thyroxine dehalogenase

The enzyme was found in a variety of non-thyroidal tissues from every species of animal studied (Table 5). All extracts were prepared according to the method described for the first step for the rabbit-muscle enzyme. It is likely that more than one mechanism operated for the deiodination of thyroxine in tissues such as liver and kidney. The deiodination, due to an enzyme of the type of thyroxine dehalogenase in rabbit muscle, was therefore estimated from the control incubation in the presence of mM-atebrin and 0·5 mM-8-hydroxyquinoline, which have been shown to inhibit the rabbit-muscle enzyme (Tata, 1959*c*). The values in Table 5 are only approximate since with liver and kidney the products formed by the action of other enzymes on thyroxine could also have been substrates for the dehalogenase. A further complication was that the contamination of

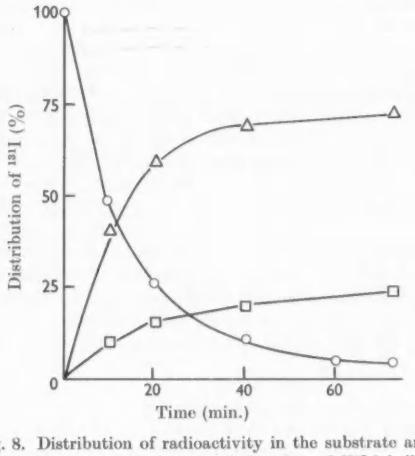


Fig. 8. Distribution of radioactivity in the substrate and the products formed during deiodination of ^{131}I-labelled L-thyroxine. Thyroxine dehalogenase (12 mg., 72-fold purification), 6·5 μm-moles of L-T_4, 0·05 μg. ion of Fe^{2+}, 0·1 μmole of FMN in 2·7 ml. of tris–HCl buffer, pH 7·0. ○, Thyroxine; △, iodide; □, iodinated protein.

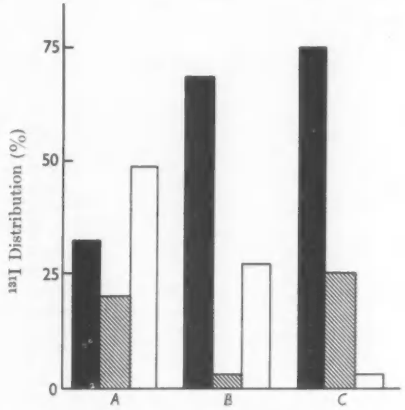


Fig. 9. Effect of activators when added separately or together on the nature of radioactive products formed at the end of incubation for 60 min. of ^{131}I-labelled L-thyroxine with the enzyme. Conditions are as in Fig. 8. □, Thyroxine; ■, iodide; ▨, iodinated protein. *A*, 0·1 μmole of FMN alone; *B*, 0·05 μg. ion of Fe^{2+} alone; *C*, 0·1 μmole of FMN and 0·05 μg. ion of Fe^{2+} added together.

extracts of more vascular tissues such as liver and kidney with serum proteins would cause an inhibition of the enzyme due to thyroxine-binding, as shown above, and hence tend to give lower values. Allowing for these considerations, liver and kidney possessed more thyroxine dehalogenase than skeletal muscle in any species. Also the muscles of both sea-water fish (plaice) and fresh-water fish (trout) were relatively richer in thyroxine dehalogenase than was the mammalian tissue.

DISCUSSION

The enzyme properties described above and those described by Tata (1959*c*) show clearly that the thyroxine dehalogenase of skeletal muscle is different from the iodotyrosine-deiodinating systems in the thyroid and liver described elsewhere (Roche *et al.* 1952, 1953; Tong, Taurog & Chaikoff, 1954; Stanbury, 1957; Stanbury & Morris, 1958; Lissitzky, Bénévent, Roques & Roche, 1959; J. Wolff, personal communication). The principal points of difference are summarized in Table 6. The differences in cofactors required and the preference shown for the different substrates are striking; the exactly opposite order of affinity for iodothyronines and iodotyrosines exhibited by the two types of enzymes is an interesting phenomenon. It should, however, be pointed out, as the work of Tong *et al.* (1954) and Nakano & Danowski (1959) on the deiodination of iodotyrosine by liver and thyroid preparations has shown, that the deiodination of iodotyrosines might involve different mechanisms or different enzymes in the two tissues. Thyroxine dehalogenase does not possess the same properties as either the 'N' or 'R' systems of Sprott & Maclagan (1955). It may, however, be present in preparations deiodinating thyroxine described by others (Maclagan & Reid, 1957; Plaskett, 1958; Yamazaki & Slingerland, 1959; J. B. Stanbury personal communication), although the data available for comparison are limited and some properties are not identical.

Table 5. *Presence of thyroxine dehalogenase in different tissues of a variety of species of animals*

'Supernatant' extracts were prepared in tris–HCl–KCl buffer as for rabbit skeletal muscle. All enzyme assays were performed in the presence of 10 μM-FMN and 6 μM-Fe^{2+} ion; in control experiments 10 μM-atebrin and mM-8-hydroxyquinoline were added; incubation was for 15 min.

Species	Tissue	T_4 dehalogenase (units/mg. of protein)
Man	Muscle	0·58
	Liver	1·86
Rabbit	Muscle	0·46
	Liver	3·01
	Kidney	2·15
	Brain	0·29
Rat	Muscle	0·68
	Liver	4·12
	Kidney	2·06
	Brain	0·37
Chicken	Muscle	0·70
	Liver	4·55
	Brain	0·52
Frog (*Rana pipiens*)	Muscle	0·40
	Liver	2·66
Trout (*Salmo fario*)	Muscle	1·95
	Liver	3·30
Plaice (*Pleuronectes platessa*)	Muscle	1·70
	Liver	3·72

The partial purification of the enzyme has been based on classical and simple methods of protein fractionation giving preparations sufficiently rich in thyroxine dehalogenase for most studies of its properties. The unusual feature of the purification procedure lies in the first step, where the enzyme could only be extracted in the 'soluble' form by using a medium of relatively high ionic strength. This, together with its solubility in water in the later stages, suggests an initial association with material of low solubility or even with particulate matter.

The higher pH optimum observed for tri-iodothyronine as substrate as compared with thyroxine (Fig. 3) was also encountered with other halogenated mono- and di-substituted phenolic derivatives. The uniform increment of about 1 pH unit for *ortho*-monosubstituted phenols could be due to the fact that the pH optimum depends on the activation of both the active group of the enzyme and the substrate. The ionization of the phenolic hydroxyl group of these compounds may be involved in the activation of the substrate; the partial pK values for the phenolic hydroxyl group are in the region of 6·5 for *ortho*-disubstituted and 8·0 for the *ortho*-monosubstituted thyronines and

Table 6. *Differences in the properties of thyroxine dehalogenase in muscle and the iodotyrosine deiodinase of thyroid and liver*

Enzyme	Tissue	Cellular fraction	Cofactor requirement	Relative substrate affinity
Thyroxine dehalogenase	Muscle	'Soluble'	Fe^{2+}, flavin	$T_4 > T_3 >$ DIT $>$ MIT
Iodotyrosine deiodinase	Thyroid Liver	Microsomes	TPNH	MIT $>$ DIT $> T_3 > T_4$

tyrosines. It is relevant to consider that the ionization of the phenolic hydroxyl group has been shown to have a profound influence on some of the physicochemical properties of thyroxine, tri-iodothyronine and related substances (Gemmill, 1955; Tata, 1959*a*).

The following generalizations may be made from the K_m values in Table 3: (*a*) thyronine derivatives are dehalogenated more readily than tyrosine derivatives, (*b*) iodinated derivatives are dehalogenated more rapidly than brominated derivatives, and (*c*) *ortho*-disubstituted phenols react more rapidly than *ortho*-monosubstituted phenols.

The substrate specificity (Fig. 4 and Table 3) of the enzyme raises some points of physiological significance. The lower affinity of the enzyme for tri-iodothyronine as compared with thyroxine appears at first to be incompatible with the higher rate of deiodination of tri-iodothyronine observed *in vivo* (see Pitt-Rivers & Tata, 1959). This apparent anomaly can be explained by the recent studies of Tata & Shellabarger (1959), who demonstrated that the shorter biological half-life of tri-iodothyronine in mammals could be accounted for by the greater ease with which this hormone can diffuse from blood into tissues. The extremely small amounts of both hormones that would be found in tissues with a relative excess of the dehalogenase means that enzymic dehalogenation is not rate-limiting in the intact animal. Thus the more rapid deiodination of tri-iodothyronine *in vivo* would only be a consequence of its more rapid diffusion into tissue cells. The relatively low affinity of thyroxine dehalogenase for iodotyrosines suggests that it is not the major enzyme involved in the extrathyroidal deiodination of iodotyrosines, especially as a powerful iodotyrosine-deiodinating system of a different nature has been demonstrated in the liver (Roche *et al.* 1952, 1953; Tong *et al.* 1954; Hartmann, 1955; Stanbury, 1957; Stanbury & Morris, 1958). A change in the optical activity of thyroxine did not markedly modify the high affinity of the enzyme for thyroxine. This, at first, might appear to be at variance with the recent findings of Larson, Tomita & Albright (1959) that D-thyroxine did not undergo deiodination to 3:5:3′-tri-iodothyronine under conditions in which L-thyroxine was partially deiodinated. However, a comparison of their results with those reported here is not justified since no indication is given in their work about the amount of iodide formed from D-thyroxine.

Probably the most interesting property of the enzyme is its resemblance to serum thyroxine-binding protein in its relative affinities for thyroxine and related compounds, as shown in Table 7. Such a resemblance suggests that the binding sites or active centres of the two proteins (one extracellular, the other intracellular) are probably alike. Competition for thyroxine thus exists when the dehalogenase and serum thyroxine-binding proteins are present simultaneously and consequently the enzymic dehalogenation is retarded in the presence of serum (Figs. 5, 6; Table 4). An important feature is the suppression of deiodination of thyroxine by the addition of a protein fraction (C-TBP in Table 4) also extracted from rabbit skeletal muscle and possessing thyroxine-binding properties. This competition of two (or more) proteins present in the same cell for thyroxine recalls the suggestion already made by the author (Tata, 1958*a*) that the amount of the hormone available for utilization in any tissue is determined by the affinity and capacity of various intracellular components that bind thyroxine. This concept has recently received some support from the work of Lissitzky, Roques & Bénévent (1959), although their 'supernatant' fraction was likely to contain rabbit-serum thyroxine-binding protein.

It is clear from these results that thyroxine dehalogenase is capable of interacting only with free or non-protein-linked substrate. This property can be used in studying the interaction between thyroxine and thyroxine-binding proteins, as explained below.

Table 7. *Relative affinities of thyroxine dehalogenase and human-serum thyroxine-binding protein in interaction with thyroxine and compounds related to it*

Values for L-thyroxine are taken as 100.

Substance	Relative affinity of T_4 dehalogenase*	Relative affinity of serum thyroxine-binding protein
L-Thyroxine	100	100
D-Thyroxine	71	80
3:5:3′-Tri-iodo-L-thyronine	22	25–30
3:5-Di-iodo-L-tyrosine	6	<5
3:5:3′:5′-Tetrabromo-DL-thyronine	18	20
3:5:3′-Tribromo-DL-thyronine	4	7
3:5-Dibromo-L-tyrosine	2	<3

* Calculated from K_m values in Table 3.

Since the protein-binding of thyroxine is a phenomenon of simple equilibrium, the level of free thyroxine

$$[T_4] = \frac{K \times [TBP\text{-}T_4]}{[TBP]}, \quad (1)$$

where K is the dissociation constant and [TBP-T_4] and [TBP] the concentrations of complexed and free binding-protein respectively. From the laws of enzyme kinetics initial rate of deiodination,

$$v = \frac{V}{1 + (K_m/[T_4])}, \quad (2)$$

where V is maximum rate of deiodination, K_m is Michaelis constant of the enzyme.

Combining the two equations,

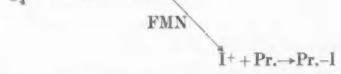
$$v = \frac{V \times [TBP]}{1 + \{K_m/(K \times [TBP\text{-}T_4])\}}. \quad (3)$$

(The term 'TBP' can in fact represent any thyroxine-binding substance). Thus the measurement of initial rates of deiodination of thyroxine in the presence of different amounts of binding proteins would indicate the dissociation constant for the protein–thyroxine complex.

The study of the nature of products formed was limited by the fact that only the more labile halogen atoms in *ortho* position to the phenolic hydroxyl group were labelled. The formation of iodide as the major product of dehalogenation of thyroid hormones, their acetic acid analogues and iodotyrosines is compatible with the urinary excretion of iodide as the major product of metabolism of these substances *in vivo* (see Pitt-Rivers & Tata, 1959; Roche, Michel, Jouan & Wolf, 1956; Wilkinson, 1959). On the other hand, the iodination of proteins observed in these experiments has not been described in studies on metabolism of thyroid hormones in the intact animal. The minute amounts of iodine that would be involved *in vivo* in the presence of relatively vast amounts of tissue proteins would make it impossible of detection by ordinary methods of chromatography. In one experiment it was possible to isolate iodinated insulin when insulin was added in sufficient amounts (5–10 mg./ml.) during the deiodination of thyroxine (Tata, 1960*c*). (Insulin was used not only because it was a protein foreign to skeletal muscle but also because of its low thyroxine-binding power and the ease in distinguishing it from muscle proteins by electrophoresis.) It would be interesting to see whether pure thyroxine dehalogenase would iodinate or brominate itself during the dehalogenation of its substrates. The overall transfer of halogen atoms from thyroxine and other halogenated phenols to the tyrosyl radicals of protein can be termed 'transhalogenation'. This reaction is not peroxidative and is hence totally different from the 'transiodination' mediated by horseradish peroxidase and described by Saunders & Stark (1958). The almost obligatory requirement for flavin in the simultaneous iodination of proteins (Fig. 9) sheds some light on the possible dehalogenation mechanisms involved. Three possibilities exist: (*a*) thyroxine dehalogenase is composed of two enzymes, the first Fe^{2+} ion-dependent and catalysing deiodination, the second flavin-dependent, catalysing the oxidation of I^- and consequently leading to iodination of proteins:

$$T_4 \xrightarrow{\text{Enzyme } (Fe^{2+})} I^- + X \xrightarrow{\text{Enzyme (FMN)}} I^+ \rightarrow \text{Pr.–I},$$

where X is deiodinated residue of thyroxine; Pr.–I, iodinated protein; (*b*) it is one enzyme with two active centres, one dependent on Fe^{2+} ions and the other on flavin (the same enzyme would act as both a deiodinase and an iodide-oxidizing enzyme); (*c*) both the flavin-dependent and Fe^{2+} ion-dependent sites of the same enzyme attack the original substrate but release the halogen in different states of oxidation so that only the atoms released by the flavin centre can take part in the substitution of tyrosyl radicals:



'I^+' in the above schemes denotes any one or more oxidized states of iodine participating in the iodination of proteins whereas the form $[I]_a$ is not available for this reaction. From the facts that the same ratio of specific enzyme activities, when assayed in the presence or absence of FMN and Fe^{2+} ions, was found at different steps of purification and that addition of flavin after most of the substrate had been deiodinated by the Fe^{2+} ion-activated enzyme failed to iodinate the proteins present, the latter possibility appears the most likely; confirmation must, however, await purification of the enzyme.

Some deiodination accompanies the oxidative action of polyphenoloxidase (Lissitzky & Bouchilloux, 1957) or the non-enzymic action of a Fe^{2+} ion–ascorbate system (Lissitzky & Roques, 1957) on some iodotyrosines and iodothyronines. The nature of products obtained with thyroxine dehalogenase and other properties of the enzyme rule out a similar mechanism in this case. Soodak & Maloof (1959) have found that the effect of flavin stimulation of iodoprotein formation reported earlier (Tong, Taurog & Chaikoff, 1957) may be non-enzymic and based on photochemical oxidation of iodide by flavin derivatives.

In line with previous reports (Tata *et al.* 1957; Tata, 1957, 1958*b*), 3:5:3′-tri-iodothyronine was not a consistent product of deiodination of thyroxine, even with short incubation periods and low concentrations of the enzyme. This suggests that the enzyme catalyses the simultaneous removal of the two iodine atoms in the *ortho* position to the phenolic hydroxyl group. The failure to detect partial dehalogenation is in contrast with the reports of Larson, Albright and colleagues about the conversion of thyroxine into tri-iodothyronine *in vitro* (Albright *et al.* 1954; Albright & Larson, 1959; Larson *et al.* 1955, 1959). The conversion appears only to be a property of intact kidney slices. The majority of recent publications on the enzymic deiodination of thyroxine, however, also report failure to detect the formation of tri-iodothyronine (Yamazaki & Slingerland, 1959; Lissitzky, Bénévent, Roques & Roche, 1959; Etling & Barker, 1959*a*). On a physiological basis it can be concluded that thyroxine dehalogenase, as studied above, is not involved in the 'activation' of thyroxine to a more potent substance.

Further knowledge of the mechanism of action of the enzyme requires its more complete purification. Also, the experiments recorded in this paper reveal little about the physiological role of thyroxine dehalogenase; studies of this enzyme in health and disease, and in different states of thyroid function and metabolic activity, are desirable.

SUMMARY

1. A method for a 60–90-fold purification of the enzyme, thyroxine dehalogenase from rabbit skeletal muscle, has been described. Enzymic deiodination and debromination of ^{131}I- and ^{82}Br-labelled thyroxine and some iodo- and bromothyronines, thyroacetic acids and tyrosines have been described.

2. The enzyme was unstable on storage and was not resistant to freezing and thawing or to freeze-drying.

3. Thyroxine dehalogenase was activated by Fe^{2+} ions and by flavins (flavin mononucleotide, flavinadenine dinucleotide and riboflavin); the activation by these factors appears to be additive, but the activators acting singly lead to formation of different products.

4. Relationship between amount of enzyme and initial velocity of deiodination was linear only if a correction were applied for the 'instability' of thyroxine observed with small amounts of the enzyme.

5. Inhibition of the enzyme by Hg^{2+} ions could be reversed to about 40 % of the original activity by the addition of excess of flavin mononucleotide.

6. The pH optima were around 6·4 for *o*-dihalogenated phenols and around 7·3 for *o*-monohalogenated phenols.

7. Of all the compounds tested the enzyme had the highest affinity for thyroxine, which was about four to five times that for tri-iodothyronine and 20 times that for 3:5-di-iodotyrosine. D-Thyroxine was readily deiodinated. K_m values have been given for some iodinated and brominated thyronines, thyroacetic acids and tyrosines. The relative affinities of thyroxine dehalogenase for thyroxine and related compounds closely resemble those of serum thyroxine-binding protein.

8. Addition of blood serum suppressed enzymic dehalogenation of thyroxine. The extent of inhibition thus caused was equal to the fraction of thyroxine bound to serum thyroxine-binding protein and albumin. Thyroxine dehalogenase can attack only free or non-protein-linked substrate. A new method based on this property for studying binding of thyroxine to proteins is proposed.

9. The major product of deiodination was iodide. About 20–25 % of the iodine released was incorporated into proteins present in the enzyme preparation; the formation of iodinated proteins required flavin but not Fe^{2+} ions. 3:5:3′-Tri-iodothyronine was not one of the important products of deiodination of thyroxine.

10. The findings are discussed in the light of enzyme mechanism and some physiological implications.

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Biochem. J. (1960) **77**, 226

The Phospholipids of the Erythrocyte 'Ghosts' of Various Species

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There is abundant biological evidence of an active transport of cations across cell membranes (e.g. Hodgkin, 1951). Active transport is most easily explained by supposing a carrier molecule within the membrane, and an important task in biochemistry is the identification of such a carrier. A number of authors have suggested that one or more of the kephalins would fit the role (e.g. Davies & Krebs, 1952). Solomon, Lionetti & Curran (1956) found that chloroform–methanol extracts of human blood contained a much higher potassium/sodium ratio than was to be expected from the concentration of these ions in the original blood; on shaking the extract with water the ions were released. With purified phospholipid preparations, phosphatidylserine was particularly effective in taking up potassium relative to sodium. Kirschner (1959) attempted a crude fractionation of the phospholipids of pig erythrocytes. One fraction, which he tentatively identified as phosphatidylserine, contained sodium/potassium in the ratio 1·6/1, although the internal cation of pig erythrocytes is almost entirely potassium. Recently Hoffman, Schulman & Eden (1959) have described a model system in which phosphatidylethanolamine could specifically transport sodium across an artificial membrane.

Vogt (1957) has suggested phosphatidic acid as a possible cation carrier, and this view is supported by Hokin & Hokin (1959), who showed that slices

of the salt gland of the albatross (which is controlled by cholinergic nerves), when treated with acetylcholine, showed an increase over controls in the incorporation of ^{32}P into phosphatidic acid, whereas the incorporation into other phospholipids was unchanged.

In order to function as a carrier, a suitable phospholipid need be present in cell membranes in small quantities only, if the cation-exchange rate is sufficient. For most tissues it is impossible to prepare membranes freed from other cellular material, but erythrocyte stromata may approximate to this (Davson, 1959). For this reason we examined the composition of stromata and found the ratio of phosphatidylserine/phosphatidylcholine was high in ruminant stromata (noted by Dawson, 1957). While this work was in progress Turner (1957) published a preliminary account of semi-quantitative studies on the phospholipid composition of erythrocyte stromata and noted the absence of lecithin in ruminants, in contrast with non-ruminants. Similar results have recently been reported by Hanahan, Papahadjopoulos & Feminella (1959).

We have therefore extended our studies over a number of species, with a recently developed method (Dawson, 1960) for the analysis of individual phospholipids. This method allows greater discrimination between the acidic kephalins, which might be involved in cation transport.

EXPERIMENTAL

Preparation of stromata. Blood was obtained by venepuncture, except for pig blood, which was obtained immediately *post mortem*, and human blood, for which outdated transfusion blood was used. Heparin was used as anti-coagulant.

In the preparation of stromata, the erythrocytes were separated from plasma by centrifuging for 15 min. at 1200 *g*. The erythrocytes were washed three times in aq. 0·9% sodium chloride, and then haemolysed with an equal volume of water. The stromata were separated in a Sharples centrifuge at 12 000 *g* and washed thoroughly with water. On examination under the microscope they gave the typical appearance of erythrocyte 'ghosts'. When whole erythrocytes were used, these were washed only once in aq. 0·9% sodium chloride.

Preparation and analysis of lipid extracts. If the suspension of erythrocyte 'ghosts' or whole cells was directly treated with a chloroform–methanol mixture (2·1, v/v) according to the extraction procedure of Folch, Lees & Sloane-Stanley (1957), it congealed into a plastic putty-like lump. Consequently the suspension was first blended mechanically with 7 vol. of methanol, and then 14 vol. of chloroform was added. This resulted in a fine suspension which, after standing for 10–15 min., was either filtered through a coarse paper (Whatman no. 531) or centrifuged. The extract was shaken with 0·2 vol. of aq. 0·85% sodium chloride solution to remove non-lipid contaminants and allowed to stand at 2° until the solvent layers separated. The lower layer was withdrawn, filtered and concentrated to about one-fifth on a rotary vacuum dryer. Lipid extracts of plasma were obtained in a similar manner.

The individual phospholipids in the complex lipid mixture extracted were estimated by the successive hydrolysis procedure of Dawson (1960), each determination being done in duplicate. Total phospholipid phosphorus was determined by the procedure of Fiske & Subbarow (1925) after the lipid residue had been digested with perchloric acid.

Phosphatidic acid. Sodium phosphatidate was prepared enzymically from ovolecithin. The phospholipase D employed was prepared by homogenizing the yellow hearts of Brussels sprouts with 0·1 M-sodium acetate–acetic acid buffer, pH 5·6, and incubating this with lecithin according to the method of Davidson & Long (1958). After incubation the phosphatidic acid was extracted from the reaction mixture with ether, washed with 0·1 N-HCl and then purified by the method of Kates (1955). However, for removing traces of pigment it was found better to precipitate the sodium phosphatidate from ether with ethanol rather than with acetone.

On deacylation by mild alkaline hydrolysis (Dawson, 1960), 98% of the phosphorus was recovered as glycerophosphoric acid; no phosphodiesters were produced.

RESULTS

The results of the analysis of the phospholipids of erythrocyte 'ghosts' are shown in Table 1. Values for the recoveries show that approximately 91% of the lipid phosphorus was accounted for in the fractions examined, with individual total recoveries varying from 83 to 102%. The reason for this is unknown and it is in contrast with the more quantitative recoveries (94–104%) obtained for the plasma phospholipids (Table 2) and also of the phospholipids of sheep tissues found in a previous investigation (Dawson, 1960). There is a striking difference between the percentages of lecithin in the erythrocyte 'ghosts' of ruminants (mean ± S.E.M. = 9·1 ± 1·6) and those of non-ruminants (31·7 ± 2·2), a difference which is highly significant ($P < 0·001$). This lack of choline in the form of lecithin in ruminant-erythrocyte 'ghosts' is almost exactly compensated for by larger percentages of the other choline-containing phospholipids, i.e. sphingomyelin and choline plasmalogen. [Mean percentages: sphingomyelin (ruminants) 37·7 ± 2·5, (non-ruminants) 19·8 ± 2·9, $P < 0·001$; choline plasmalogen (ruminants) 7·6 ± 0·6, (non-ruminants) 4·9 ± 0·8, $P = 0·01$.]

The percentage of phosphatidylethanolamine appears to be much higher in the omnivores (man and pig) than in the herbivores (horse, cow, sheep and goat), the difference being statistically significant (herbivores 7·1% ± 0·8; omnivores 20·4 ± 3·5, $P < 0·01$). On the other hand, phosphatidylserine shows no significant variation between ruminants (13·6 ± 0·5) and non-ruminants (12·4 ± 1·5), and

this also applies to the minor phospholipid components.

The species variations seen in the phospholipids of erythrocyte 'ghosts' are in no way reflected in the distribution of individual phospholipids in the blood plasma (Table 2). Choline-containing phospholipids accounted for 94–98 % of the lipid phosphorus recovered.

In three cases where whole erythrocytes were examined (Table 1), there was no obvious difference from the distribution found in the 'ghosts'. That this is not simply because all the phospholipids of erythrocytes sediment on the centrifuging was shown by an experiment with haemolysed pig erythrocytes, where 41 % of the lipid phosphorus was found not to sediment on centrifuging at 36 000–90 000 ***g*** for 90 min.

During the washing procedure used for purifying lipid extracts there is a tendency for small amounts of acidic phospholipids to be lost in the washing media. Consequently phosphatidic acid (7 %) was added to a chloroform–methanol extract of the lipids of sheep erythrocytes, and after washing with aq. 0·85 % sodium chloride the chloroform layer was analysed for phospholipids. About 80 % of the added phosphatidic acid was recovered, which suggests that any losses of phosphatidic acid occurring are small and unlikely to affect the analytical conclusions reached in this investigation.

DISCUSSION

Although the osmotically disrupted blood cells used in the present work were not entirely erythrocytes, it is unlikely that their analyses would be substantially different from the corresponding values for pure erythrocytes, because of the overwhelming preponderance of these cells in normal blood. It cannot, however, be assumed that the erythrocyte-'ghost' preparation represents only the membrane of the cell for it is possible that it contains other structures which in the original cell carried out functions apart from ion and metabolite transport.

Any theory of cation transport involving the hypothesis of a carrier does not necessarily require that the latter should be present in high concentrations. Nevertheless, it is interesting to look at the present results in the light of past suggestions about the kephalin nature of such a carrier. There is no indication that phosphatidic acid is present as more than a very minor part of the phospholipid fraction. On the other hand, phosphatidylserine is present in the phospholipids of erythrocyte 'ghosts' at a concentration which is probably higher than that in most other tissues apart from the nervous system. Its concentration is, moreover, generally constant in the erythrocytes of all

Table 1. *Phospholipids in whole erythrocytes and 'ghosts'*

Results express the phosphorus of phospholipids as a percentage of the total lipid phosphorus. Each value represents the mean of a duplicate determination on a different preparation of 'ghosts' or of whole cells.

	Human			Pig,	Horse,	Cow			Sheep			Goat,
	'Ghosts'		Whole cells	'Ghosts'	'Ghosts'	'Ghosts'		Whole cells	'Ghosts'		Whole cells	'Ghosts'
Lecithin (+lysolecithin)	28·2	30·3	36·4	25·1	37·1	14·1	5·5	7·0	11·0	4·4	2·4	7·5
Phosphatidylethanolamine	13·6	15·2	17·6	27·7	9·4	11·4	6·7	6·2	6·1	4·8	4·1	6·3
Phosphatidylserine	10·4	13·0	5·1	18·6	9·5	12·8	15·2	15·4	12·4	12·9	13·6	14·8
Sphingomyelin	15·8	19·3	16·2	15·4	23·8	31·2	48·2	39·2	37·0	48·0	—	33·1
Choline plasmalogen	5·3	2·3	5·7	5·3	7·9	7·8	6·2	5·3	10·2	7·8	—	6·7
Ethanolamine plasmalogen	2·1	6·0	5·6	1·5	4·2	3·3	2·1	1·1	3·4	0·8	—	3·2
Serine plasmalogen	0·8	0·8	0·3	1·5	0	1·2	0	2·2	1·7	0·5	—	2·3
Monophosphoinositide	1·8	1·4	0	3·5	4·8	3·8	3·2	1·8	3·5	3·8	2·2	6·4
Phosphatidic acid	1·2	1·7	1·2	0·9	1·6	0·7	0·5	0·3	0·5	0·3	0	0
Polyglycerolphospholipid	0	0	0	0	0·9	1·0	0	0·3	0·8	0	0·4	1·9
Unknown	3·5	3·0	2·6	0·9	2·5	2·8	2·4	1·1	3·5	1·6	—	1·8
Recovery	82·7	93·0	90·7	100·4	101·7	90·1	90·0	79·9	90·1	84·9	—	84·0

Table 2. *Phospholipid in plasma of various species*

Results express the phosphorus of phospholipid as a percentage of the total lipid phosphorus.

	Human	Pig	Horse	Cow	Sheep	Goat
Lecithin (+lysolecithin)	74·5	79·0	83·2	82·0	70·3	88·0
Phosphatidylethanolamine	1·8	2·2	0·5	0	2·4	0·5
Phosphatidylserine	0	0	0	0	0	0
Sphingomyelin	12·4	10·3	10·4	10·8	15·8	9·0
Choline plasmalogen	3·8	2·7	1·5	6·4	2·1	2·0
Ethanolamine plasmalogen	0	0	1·0	0	0	0
Serine plasmalogen	0	0	0	0	0	0
Monophosphoinositide	1·4	2·2	0	2·4	0	1·7
Phosphatidic acid	0	0	0	0	0·9	0·4
Polyglycerolphospholipid	0	0·5	0	0·7	1·6	0
Unknown	1·3	0·6	0·7	2·0	0·6	0·8
Recovery	95·2	97·5	97·3	104·3	93·7	102·4

species, in contrast with the large species variations found in the concentrations of lecithin and phosphatidylethanolamine.

Another acidic phosphoglyceride which could conceivably function as a cation carrier, namely monophosphoinositide, was present in small but definite amounts. It was present in highest concentration in the erythrocyte 'ghosts' of the goat and was lowest in those of man. Bangham, Pethica & Seaman (1958) have described the charged groups at the surface of a sheep erythrocyte as having properties consistent with those of a phosphate and it seems likely that these could result from the presence of monophosphoinositide and, to a much lesser extent, polyglycerolphospholipid and phosphatidic acid.

The present results on the phospholipid analysis of whole erythrocytes and their 'ghosts' confirm and extend previous reports of the high phosphatidylserine content of the phosphoglyceride fraction of ruminant erythrocytes (Dawson, 1957), and the very small amounts of lecithin in ruminant erythrocytes compared with those in erythrocytes of the non-ruminant (Turner, 1957; Turner, Anderson & Gandal, 1958; Hanahan *et al.* 1959). They also reveal other species differences, namely an increase in the percentage of sphingomyelin in the ruminant-erythrocyte 'ghosts' compared with those of the non-ruminant. Moreover it is apparent that in the omnivores (pig and man) the concentration of phosphatidylethanolamine in the 'ghosts' is many times greater than the concentration in those of the strict herbivores, i.e. horse, sheep, cow and goat. In the pig erythrocyte 'ghosts' its concentration is as high as that of the lecithin fraction, which is an unusual finding for mammalian tissues.

The reason why the erythrocyte 'ghosts' of ruminants possess so much less lecithin and phosphatidylethanolamine and more sphingomyelin than those of non-ruminants is not obvious. It is pertinent perhaps to mention here that ruminant erythrocytes are much smaller than those of the non-ruminant and that they are, in contrast with those of the non-ruminant, almost devoid of diphosphoglycerate (Rapaport & Guest, 1941).

The species differences of erythrocyte phospholipids are not in any way reflected in the relative concentrations found in the plasma. In all species, over 94% of the phospholipid fraction can be accounted for as the choline-containing phospholipids, lecithin + lysolecithin, choline plasmalogen and sphingomyelin. This is in agreement with a number of previous reports of the complete dominance of the choline-containing phospholipids in the plasma of various species (e.g. Sinclair, 1948; Axelrod, Reichenthal & Brodie, 1953), and extends these findings to the horse, sheep and cow. Even the small amounts of kephalin present may possibly arise from chylomicrons, which are difficult to remove from the plasma by centrifuging and which are known to contain kephalin (Robinson & Poole, 1956).

Turner *et al.* (1958) have suggested that the lack of lecithin in washed ruminant erythrocytes may explain their relative resistance to haemolysis by snake venoms and the α toxin of *Clostridium welchii*. The present findings, in confirming the lower concentration of lecithin in ruminant-erythrocyte 'ghosts', would agree with this viewpoint, although, in contrast with the results of Turner *et al.* (1958), some lecithin was always found. It is unlikely that this represents trapped plasma lecithin, since ruminant whole cells, which were less efficiently washed than the 'ghosts', contain no more lecithin. Nevertheless, the amount of haemolytic lysolecithin formed by cobra venom and the disruptive effect of α toxin are likely to be much less with ruminant erythrocytes than in the non-ruminant erythrocytes.

SUMMARY

1. The composition of the phospholipid of erythrocyte 'ghosts' and plasma from six species (man, pig, horse, cow, sheep and goat) has been measured.

2. The percentage of lecithin in the non-ruminant 'ghosts' was over three times as high as that found in ruminant 'ghosts'.

3. In contrast, the concentrations of sphingomyelin and choline plasmalogen were higher in ruminant 'ghosts' and consequently there were no significant differences between the concentrations of total choline-containing phospholipid in the various species.

4. Phosphatidylethanolamine was markedly higher in the 'ghosts' of omnivores than in 'ghosts' of herbivores.

5. Choline-containing phospholipids (mainly lecithin) accounted for over 94% of the plasma phospholipid of all species.

6. Of the possible acidic phospholipids in erythrocyte 'ghosts' which could function as cation carriers, phosphatidylserine was present at a relatively high concentration (8·9–16%) in all species, phosphatidylinositol varied from 1·6% in man to 5·8% in the goat and phosphatidic acid was below 1·5% in all species.

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Biochem. J. (1960) **77**, 230

Evidence for the Chemical Interaction of Urease in Solution

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Urease prepared by the method of Sumner (1926) and recrystallized by the modified procedure of Dounce (1941) has in all cases reported shown at least two peaks in the ultracentrifuge (e.g. Sumner, Gralen & Eriksson-Quensel, 1938; McLaren, Sheppard & Wagman, 1948). Enzymic-activity studies (Hofstee, 1949), sedimentation and activity studies (Kuff, Hogeboom & Striebich, 1955), molecular-weight estimations (Setlow, 1952) and kinetic experiments (Kistiakowsky & Lumry, 1949; Ambrose, Kistiakowsky & Kridl, 1950) all provided results which could be interpreted to some degree by assuming that the components observed in the ultracentrifuge are aggregates of the same species. In this connexion it is pertinent that a reaction involving the readily detectable sulphydryl groups in the undenatured enzyme (Hellerman, Chinard & Dietz, 1943) to form disulphide linkages has been discussed (Sizer & Tytell, 1941; Kistiakowsky & Lumry, 1949); this disulphide-bond formation may occur intermolecularly, as such a cross-linkage has been postulated with other protein systems where sulphydryl groups are readily 'available' (e.g., Huggins, Tapley & Jensen, 1951; Steinrauf & Dandliker, 1958). Since an understanding of the nature of this aggregation reaction, if it occurs, is essential to proper formulation of the kinetics, and hence to any interpretation of the mechanism of urease action, a detailed study has been made of the ultracentrifugal characteristics of the substance, and is reported here.

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Secondly, in view of the known ultracentrifugal complexity, the validity of some of the primary physicochemical data for urease can be questioned. Notable in this respect are (*a*) the isoelectric point, found by a minimal solubility method (Sumner & Hand, 1929), and (*b*) the widely used molecular weight, calculated from the Svedberg expression by utilizing diffusion measurements made in the presence of sulphite ions and sedimentation results found in their absence (Sumner *et al.* 1938). We have therefore investigated the electrophoretic behaviour of urease over a wide range of pH, in order to obtain information on electrophoretic complexity and to establish fundamental data on the mobility of the substance with as high an accuracy as the material warrants. By interpolation in these results, the isoelectric point has been estimated more directly. In addition, two diffusion experiments were made to test the effect of sulphite. The physicochemical data obtained present several interesting and unusual features; when correlated with enzymic-activity measurements, they lend strong support to the hypothesis of intermolecular reaction.

EXPERIMENTAL

Materials

Enzyme preparations. The seven samples of urease used in this study were prepared by the acetone-fractionation method of Sumner (1926), from jack-bean meal obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A., and were recrystallized twice by the method of Dounce (1941). Glass-distilled water previously passed through both anionic- and cationic-exchange columns was used throughout the experiments to avoid accidental inhibition of the enzyme by traces of metal ions. The glassware was cleaned with a nitric acid–sulphuric acid bath, rinsed thoroughly and steamed (Henry & Smith, 1946; Kistiakowsky & Shaw, 1953). The specific activity of the samples prepared varied from less than 10 000 to 80 000 Sumner units (S.U.), this being quite typical of other findings (Hellerman *et al.* 1943; Evert, 1952; Kistiakowsky, Mangelsdorf, Rosenberg & Shaw, 1952). The samples, with the exception of sample 5, were stored in solution at 2–4° in the presence of cysteine hydrochloride. After 2 months the enzyme solutions developed a faint turbidity and the activity in some cases had decreased by 50% (Kistiakowsky *et al.* 1952). Consequently, after 1 month, during which time the solutions remained clear and maintained their activity reasonably well, the sample was discarded.

Buffers. The composition of the numerous buffers used in this work is given in the text. The urease was either dissolved directly in the cold buffer, or dialysis in the cold for 7 days was employed to exchange different buffers, keeping the urease in solution.

Sulphite solutions. The solutions used in the experiments performed in the presence of sulphite ions had the following composition (cf. Sumner *et al.* 1938; Epstein, 1959): 0·048 M-Na_2HPO_4, 0·021 M-KH_2PO_4, 0·031 M-Na_2SO_3, 0·013 M-$NaHSO_3$. No special precautions were taken to prevent atmospheric oxidation. The oxidation–reduction potential of the solutions was not measured and, consequently, no quantitative significance was attached to the total sulphite concentration.

Methods

Enzymic-activity measurements. The assays were performed under the standard conditions specified by Sumner & Hand (1928). The reaction was stopped after 5 min. with 2 N-HCl, and the ammonia formed was determined in accordance with the general method described by Kistiakowsky *et al.* (1952). In this case a Zeo-Karb 225 ion-exchange column was used to adsorb the NH_4^+ ions, which were eluted with KOH and the solution was diluted to 250 ml. Nessler's reagent was added and the extinction was measured with a Unicam SP. 500 spectrophotometer, after 15 min. The absolute amount of ammonia formed was obtained by reference to a standard curve. Each assay reported was performed in duplicate on at least three different dilutions of the enzyme sample, chosen so that 0·1–0·4 μmole of ammonia was produced. Each set of measurements also included blanks, in which the acid was added before the enzyme; since the buffered urea solutions (used as substrate) were freshly prepared for each set of assays, the corrections were negligible.

Electrophoresis. All electrophoresis experiments were conducted at 1–2° in a Spinco model H electrophoresis–diffusion apparatus, employing simultaneously or separately Rayleigh and schlieren optics. The former system yields a record of the refractive index versus vertical-cell co-ordinate in terms of interference fringes; the number, J, of such fringes between successive schlieren minima is accurately determinable and forms a convenient comparative unit to specify concentrations in electrophoresis experiments (Longsworth, 1951). In other aspects of the work described, the concentrations of urease were also determined by counting the fringes observed when a boundary was formed between the dialysed solution and its dialysate; for this reason all concentrations are quoted in terms of J. If the specific refractive increment of all components has the value 0·00180 dl.g.$^{-1}$ (cf. Perlman & Longsworth, 1948; Halwer, Nutting & Brice, 1951) then the relation $c = 5{\cdot}8 \times 10^{-3} J$ (where the concentration c is in g./100 ml.) may be applied to all values of J quoted in this paper (Creeth, Nichol & Winzor, 1958). In the mobility experiments, for which the 11 ml. cell was used, the ascending limb was sealed from the atmosphere to prevent flow through the cell due to the continually changing pressure head. Further, after a 4 hr. electrophoretic migration in one direction, the current was reversed for an equal period of time. At least five exposures were taken at suitable time intervals to record the progress of the boundary in each direction, and the average of the two descending mobilities (determined from the regression of the observed boundary position upon time) was taken as the mobility of the species. This technique, which is applicable only to very dilute solutions, precludes errors due to flow caused by volume changes within the electrode vessels and provides a sensitive means of detecting minor cell leaks. The maximum of the schlieren peak was used to measure the rate of movement of the boundary in mobility determinations. Since the peaks were nearly symmetrical (see below), and the concentrations low, it was considered that deter-

mination of the first moments (Longsworth & Jacobsen, 1949) would not have led to any useful increase in accuracy. The conductivities of the buffer solutions (dialysates) were used in the mobility calculations; they were measured at the temperature of the experiment, with a Philips's conductivity bridge. As the concentration of the protein used was kept at a low value (3–20 fringes), the error in assuming that the conductivities of the buffer and the protein solution are the same is small. The pH values were obtained with a Doran Universal pH meter.

Sedimentation. Sedimentation-velocity experiments were performed in a Spinco model E ultracentrifuge at 47 660 rev./min. and the sedimentation coefficients were corrected to water at 20°. The temperature was recorded, but not controlled, by the RTIC unit and the average value was taken as the temperature of the run. A variation of no more than 0·1° was observed during the normal 40 min. duration of the experiments when performed near room temperature (about 0·3° for the runs at 3°). The required viscosity and density values for the buffers were measured directly with respectively an Ostwald viscometer and a 25 ml. pycnometer. The partial specific volume of 0·73 reported by Sumner *et al.* (1938) was used in each case, assuming that it was independent of buffer composition. With systems that showed several peaks in the ultracentrifuge, the corrected areas under the peaks (Svedberg & Pedersen, 1940), together with the refractometrically determined fringe counts, were used to estimate the required concentrations. Dilutions, where necessary, were made by weight and the dilution factor was applied to the fringe count obtained on the original solution. The procedure is not valid unless all non-dialysable material is visible in sedimentation experiments during photography.

Diffusion. The diffusion experiments were performed in the Spinco model H apparatus referred to above, Rayleigh optics alone being employed. The procedure, together with a detailed discussion, has been described by Creeth *et al.* (1958). Before diffusion all protein samples (which were freshly prepared) were dialysed with periodic gentle agitation and frequent changes of buffer in the cold for 8 days. The temperature of the run was periodically recorded with a previously standardized Beckmann thermometer. The Rayleigh interferograms were measured with a two-dimensional comparator (Optical Measuring Tools Ltd., Maidenhead, Berks.), accurate to 2×10^{-4} cm., fitted with a projection screen.

RESULTS

Electrophoresis. All relevant information on the mobility experiments is summarized in Table 1; if the mobility values are plotted against pH a

Table 1. *Electrophoretic-mobility data on urease in buffers at I* 0·10 *and at* 1·0$_0$°

Sample no.	Buffer composition (M)	Concn. of protein (J)	Potential gradient (v cm.$^{-1}$)	pH	Descending mobility ($\times 10^5$, cm.^{2}/sec.$^{-1}$v^{-1})
3	0·08 NaCl 0·02 Sodium acetate 0·18 Acetic acid	5	2·95	$3·6_9$	$6·0_8$
1	0·08 NaCl 0·02 Sodium acetate 0·08 Acetic acid	3	1·98	$3·8_9$	$6·1_3$
3	0·08 NaCl 0·02 Sodium acetate 0·05 Acetic acid	5	2·93	$4·2_3$	$5·0_8$
2	0·06 NaCl 0·04 Sodium acetate 0·03 Acetic acid	3	3·22	$4·7_0$	$2·1_6$
1	0·03 NaCl 0·07 Sodium acetate 0·03 Acetic acid	5	2·48	$5·0_1$	−0·4
1	0·03 NaCl 0·07 Sodium acetate 0·01 Acetic acid	12	2·45	$5·4_6$	$-2·3_6$
2	0·08 NaCl 0·02 Sodium cacodylate 0·02 Cacodylic acid	18	3·04	$6·0_1$	$-3·2_5$
1	0·05 NaCl 0·05 Sodium cacodylate 0·01 Cacodylic acid	12	2·31	$6·6_1$	$-5·5_6$
1	0·09 NaCl 0·01 Sodium diethylbarbiturate 0·02 Diethylbarbituric acid	19	1·91	$7·4_5$	$-6·8_5$
1	0·08 NaCl 0·02 Sodium diethylbarbiturate 0·01 Diethylbarbituric acid	14	2·02	$7·9_5$	$-7·1_5$
1	0·07 NaCl 0·01 Sodium tetraborate 0·01 Boric acid	9	2·20	$8·9_0$	$-8·0_8$

smooth curve results, interpolation in which gives the value pH 5·0 ± 0·05 for the isoelectric point in sodium acetate–acetic acid–sodium chloride buffer, I 0·10. This value is in close agreement with other findings (Sumner & Hand, 1929; Wills, 1952). Anderson & Alberty (1948) reported two values of the electrophoretic mobility of urease at I 0·01; in order to compare these with the results reported here, it is necessary to use the procedure outlined by Abramson, Moyer & Gorin (1942), which is based on an inexact microscopic model of electric migration. By the use of the published diffusion coefficient (Sumner *et al.* 1938) to calculate the radius r of a hydrated sphere, $r = 61{\cdot}3 \times 10^{-8}$ cm. is obtained and, for example, the value

$$-4{\cdot}4 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{v}^{-1}$$

for the 'corrected' electrophoretic mobility at I 0·10, pH 7·4, from Anderson & Alberty's figure. By interpolation from Fig. 1, the corresponding value obtained in this work is

$$-6{\cdot}6 \times 10^{-5} \text{ cm.}^2 \text{ sec.}^{-1} \text{v}^{-1}.$$

Although these calculations are approximate, the discrepancy is large, and probably indicates greater anion-binding at the higher ionic strength.

In the electrophoresis experiments a single relatively symmetrical schlieren peak was obtained at all pH values used. The schlieren pattern shown in Fig. 1 is quite typical of results obtained, but in this case the protein concentration (J 120) is much higher than that used for mobility determinations. The limited solubility of urease at pH

Fig. 1. Electrophoresis pattern (ascending limb) of urease at pH $7{\cdot}4_9$ in diethylbarbiturate buffer, I 0·10, after 8 hr. at a potential gradient of 2·9 v cm.$^{-1}$

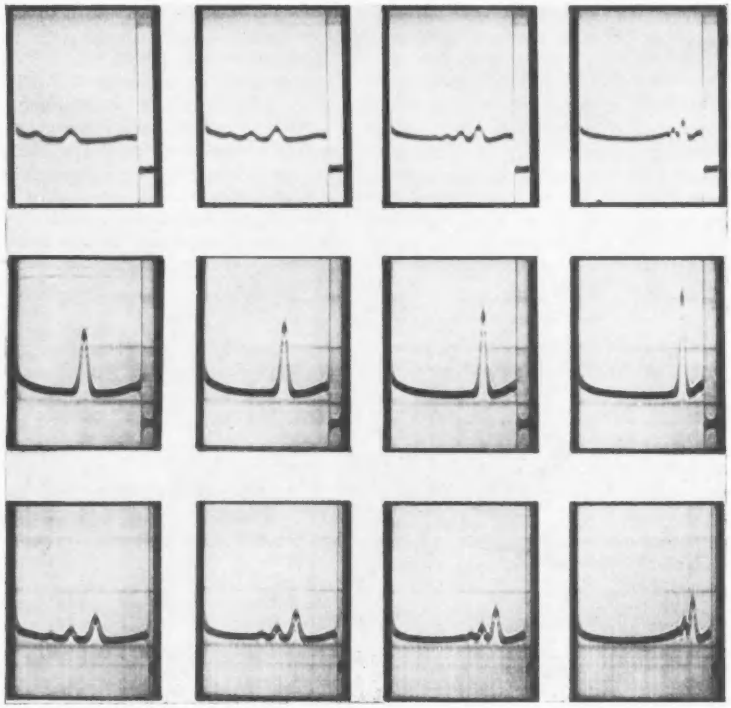

Fig. 2. Sedimentation diagrams of urease solutions in the presence and the absence of sulphite ions (sedimentation from right to left): (*a*) sample 2 in diethylbarbiturate buffer, I 0·10, pH $7{\cdot}4_5$; (*b*) sample 5 in a buffered sulphite solution, pH $7{\cdot}0_9$; (*c*) the same sample as (*b*) after dialysis with 0·96% phosphate buffer, pH $7{\cdot}0_3$.

values near the isoelectric point (Sumner & Hand, 1929; Anderson & Alberty, 1948) prevents similar analytical electrophoresis experiments being performed under these conditions. However it may be concluded from the Rayleigh interferograms that not more than 10% of another resolvable electrophoretic component was present.

Sedimentation. The patterns obtained in sedimentation-velocity experiments provide a striking comparison with those obtained in electrophoresis, where, as we have seen, the absence of appreciable amounts of components with different mobilities was demonstrated. Each sample of urease prepared was sedimented and a typical result (excluding patterns obtained with samples 4 and 5) is shown in Fig. 2 (*a*). At least four peaks are clearly visible. The S of the slowest-moving boundary could only be estimated to lie within the range 4–6 as it was diffuse and never completely resolved during an experiment. (In some experiments at low I, the S 4–6 component was not present and one with S 12 appeared.) The relative areas under the separate peaks varied considerably with different preparations; indeed with sample 4 the fastest-sedimenting boundary was completely absent, whereas with sample 5, prepared and stored in the complete absence of any protecting agent, the relative amounts of faster-sedimenting boundaries were greater than usual and the solution was slightly turbid. Consequently the electrophoresis pattern (Fig. 1) obtained with sample 5 provides a very critical test for the observed contrast between electrophoretic and sedimentation-velocity analyses. Even though the sedimentation experiments were performed at approximately 20° and the electrophoresis analyses at 1°, the comparison is justified as sedimentation of sample 7 at 3° revealed no alteration in the relative amounts of components present in comparison with a control experiment performed at 20°. The appearance of several sedimenting boundaries has been reported by all workers who have analysed urease preparations in this way (Sumner *et al.* 1938; McLaren *et al.* 1948; Kuff *et al.* 1955).

The sedimentation coefficients and the relative amounts of the three major sedimenting boundaries are given, as a function of concentration, in Table 2, where the effect of buffer type, pH and sample (referred to above) is also illustrated. The accuracy of both the estimated concentrations, and (to a lesser degree) the S values, is limited, because no account has been taken of errors due to the Johnston–Ogston effect (Johnston & Ogston, 1946) or those due to the neglect, in area determinations, of any non-dialysable material which was not clearly resolved in the photographs. However, the S values of 18·6 and 28 found by McLaren *et al.* (1948) agree within 1% with values estimated at a comparable protein concentration, after allowing for the adiabatic expansion correction of 0·9° (Biancheria & Kegeles, 1954). Nevertheless, the extrapolated $S^0_{20,w}$ value can only be given as $20 \pm 0{\cdot}5$s. The data of Sumner *et al.* (1938), although in qualitative agreement, cannot be corrected for a quantitative comparison. It is apparent, also, from Table 2 that three major sedimenting peaks are present over the range pH 6–9 and that buffer type has little effect on the S values. The increase in the relative amounts of the faster-sedimenting boundaries observed when

Table 2. *Sedimentation data on urease solutions*

Sample no.	Buffer composition (M)	pH	$S_{20,w}$* 1	$S_{20,w}$* 2	$S_{20,w}$* 3	Percentage of total area* 1	Percentage of total area* 2	Percentage of total area* 3	Concn. of I (*J*)
7	0·07 NaCl, 0·01 Sodium tetraborate, 0·01 Boric acid	8·9	19·2	28	35	73	22	5	—
2	0·09 NaCl, 0·01 Sodium diethylbarbiturate, 0·02 Diethylbarbituric acid	7·5	18·9	28	36	66	24	10	46
2			18·9	28	36	63	25	12	44
4			18·8	28	—	90	10	0	40
2			19·0	28	35	61	28	11	39
2			19·3	28	34	61	26	13	29
2			19·2	28	36	61	27	12	16
5			18·8	28	34	55	31	14	—
6	0·05 Na_2HPO_4, 0·02 KH_2PO_4	7·0	19·7	29	36	72	21	7	—
7			19·2	28	35	85	12	3	—
7†			21	31	40	83	13	4	—
2	0·08 NaCl, 0·02 Sodium cacodylate, 0·02 Cacodylic acid	6·1	19·3	28	36	60	28	12	13
2			19·6	29	—	—	—	—	10

* The resolved boundaries in Fig. 2 (*a*) are numbered (1, 2 and 3) in order of increasing sedimentation coefficient.
† This experiment was performed at 3° (all others were at ~20°): the discrepancies evident in the S_{20} values probably arise at least partly from the application of a large temperature correction factor.

sample 7 was dialysed from pH 7·0 to 8·9 cannot be taken as evidence for increased aggregation at high pH values as other variables (see below) inherent in the dialysis and handling procedures are introduced. Protein solutions dialysed to pH values near the isoelectric point, or below it, precipitated (the precipitate being insoluble in buffers at pH 7–8) and the resulting solution was too dilute for useful sedimentation-velocity analyses to be made. As is also evident from Table 2, no marked change in the relative areas under the peaks was observed on dilution; in this connexion, the insensitivity of these area determinations should be emphasized. The relative proportions of the components were also unaffected by their solutions standing for periods which ranged from 2 hr. to 30 days after the final recrystallization (for example, with sample 4, six sedimentation-velocity analyses over this period all revealed the amounts 90%, 10% and 0% for the components referred to in Table 2).

If the peaks observed in sedimentation analysis correspond directly with aggregates of well-defined molecular weight, then the effect of modifying agents [preferably group-specific reagents (cf. Putnam, 1953)] on the relative amounts of these sedimenting boundaries should be demonstrable; the experiments described below were therefore performed.

(i) Effect of sulphite ions. When buffered solutions, pH 7·0, of urease (samples 4–7) were dialysed against buffered sulphite solutions and the resulting protein solution was sedimented, the faster-sedimenting boundaries were no longer evident and the amount of the $S^0_{20,w}$ 20 boundary increased markedly. This is shown in Fig. 2 (*b*). With sample 4, this increase in area equalled, within the limits of accuracy of the determinations, the area previously under the S 28 peak, which was the only other peak visible in this sample. However, with sample 5 the increase was apparently greater than could be accounted for by the disappearance of the peaks visible in sulphite-free solutions during photography. The effect of sulphite ions was entirely reproducible with all samples tried, and reversible in the sense that when the sulphite was removed by exhaustive dialysis the other peaks returned, as is evident in Fig. 2 (*c*). The relation between overall enzymic activity of the solution and relative amounts of the separate sedimenting peaks is given in Table 3. It is apparent that an increase in activity accompanies an increase in the amount of the $S^0_{20,w}$ 20 component.

The effect of protein concentration on the S of the species existing in the presence of sulphite ions is shown graphically in Fig. 3 (data not included in Tables). The determination of concentrations was more accurate in this case as the dilutions were made by weight and thus the extrapolated $S^0_{20,w}$ value can be given as $20{\cdot}3 \pm 0{\cdot}1$ and therefore is the same within experimental error as the corresponding value obtained in the sulphite-free system. The solid line of Fig. 3 is an attempt to average the data; its curvature is unexpected, but is apparently outside experimental error.

A diffusion experiment on sample 6 (J 110·84) in a buffered sulphite solution, pH 7·0$_9$, gave the observed diffusion coefficient referred to as $\mathscr{D}_A$ (Baldwin, Dunlop & Gosting, 1955; Dunlop & Gosting, 1955) of $3{\cdot}28 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ at 20·00°, corrected to water. The sharpness of the initial boundary was judged satisfactory as the 'zero time' was 165 sec. and there appeared to be no variation of the diffusing solute with time, according to the criteria applied in a previous paper (Creeth *et al.* 1958). The value of $\mathscr{D}_A$, which agrees

Table 3. *Effect of sulphite on the enzymic activity and sedimentation-velocity patterns of urease*

The control sample was urease dissolved in veronal buffer, pH 7·5, I 0·1, stored in the cold for 1 week and diluted with phosphate buffer for assay. The sulphite-treated sample was a portion of the solution of urease in veronal buffer, dialysed for 1 week against a sulphite solution and diluted with phosphate buffer for assay. This was performed within 2 hr. of that for the control. These data were found with sample 5, which had a low specific activity (10 000 Sumner units/g.). The effect was reproduced with sample 7. The areas are denoted as in Table 2. Approx. 0·7% solution of protein was used.

	Percentage of total area			Enzymic activity of 1 ml. of solution (Sumner units)
	1	2	3	
Control	55	31	14	25
Sulphite-treated urease	100	0	0	50

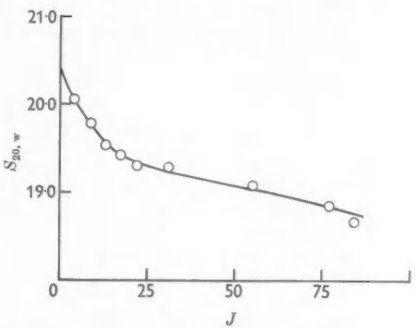

Fig. 3. Variation of sedimentation coefficient with concentration (expressed as fringes, J, see text) of the species present in a buffered sulphite solution of urease, sample 6.

with the value of $3{\cdot}46 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ found by Sumner *et al.* (1938) quite closely, is not the diffusion coefficient D_{11} appropriate to the Svedberg molecular-weight expression [cf. the exact form of this expression given by Baldwin (1958)] unless (*a*) flow-interaction effects (Baldwin *et al.* 1955; Dunlop & Gosting, 1955; Dunlop, 1957), (*b*) second-order concentration-dependence effects (Creeth, 1955; Gosting & Fujita, 1957), and (*c*) any impurities are absent. The deviation graph, Fig. 4, which gives the relative fringe deviations, Ω_R, over the whole boundary, as a function of $H(z^*)$ (Creeth, 1958), indicates clearly that some, at least, of the above conditions are not fulfilled. Although an analysis by the curve-fitting procedures described (Creeth, 1958) indicates the presence of a fast-diffusing impurity which could be tentatively associated with the S 4–6 component, such procedures are not justified where flow interactions are likely (Creeth *et al.* 1958; O'Donnell, Baldwin & Williams, 1958).

A second diffusion experiment was performed under similar conditions (veronal buffer, pH 7·5, I 0·10, J 64·66, zero time 334 sec.) but with the sulphite omitted. The results in this case were quite different, the diffusion coefficient being much lower ($\mathscr{D}_A$ $1{\cdot}8 \times 10^{-7}$ cm.^{2} sec.$^{-1}$ at 20°) and the (nearly symmetrical) deviation plot very much greater in magnitude ($\Omega_{R\ max.}$ 680×10^{-4}). The contrast in the results of these two experiments, particularly in the size of the deviation plots, is illustrative of the pronounced effect of sulphite on the system.

(ii) *Effect of cysteine hydrochloride.* This reagent was added during preparation to prevent inhibition by metal ions which could accidentally be present (Shaw, 1954). Accordingly, no fixed amount was added during the preparation of most of the samples. As has been described, the apparent proportions of the components observable in the ultracentrifuge varied widely between different samples. To test whether this variability was the direct result of varying contents of cysteine (i.e. on the assumption that cysteine and sulphite have similar actions), this reagent was not added at any stage of the preparation of sample 5. The resulting preparation had a low activity (Table 3) and relatively greater amounts of the faster-sedimenting components (Table 2). Although not conclusive, this result supports the contention that cysteine and sulphite do have similar actions on urease, in respect of reducing or reversing aggregation.

(iii) Effect of an oxidizing agent. The addition of 0·01 M-potassium ferricyanide caused immediate and complete precipitation of the protein. The product was insoluble in buffer at pH 7·0 and in this respect resembled the precipitate obtained on dialysing a urease solution to pH 5·0.

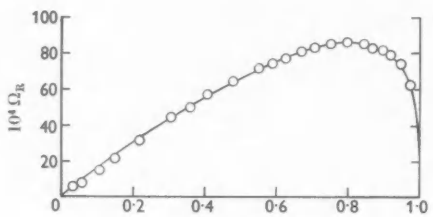

Fig. 4. Fringe-deviation diagram obtained from the diffusion of urease, sample 6, in the presence of sulphite ions at pH 7·0 .

DISCUSSION

The appearance in an electrophoresis experiment of a single peak which approximates to Gaussian form (cf. Fig. 1) cannot, of course, be interpreted as convincing evidence of electrophoretic homogeneity. Boundary-spreading experiments at the isoelectric point would be desirable, so that the extent of heterogeneity could be specified (Anderson & Alberty, 1948); these, however, are pro hibited in this case because of the low solubility. Although the mobility experiments described earlier indicate that only one peak is evident over a wide pH range, the experiments were performed at only one ionic strength and do not preclude the possibility of the presence of components with slightly differing mobilities. Nevertheless, it is clear that the marked heterogeneity of the samples in the ultracentrifuge, illustrated by Fig. 2 (*a*), cannot be directly related to the electrophoresis patterns obtained, in terms of individual components. To correlate these results, and to relate the significant finding that sulphite converts all the heavier species into one of $S^0_{20,w}$ 20, only one hypothesis is apparent, namely that urease tends to form dimers, etc., in the absence of reducing agents, these polymers having lower specific enzymic activities, but electrophoretic mobilities equal to that of the monomer. This hypothesis will now be examined in the light of all available evidence. The numerical calculations below have been made by standard procedures, following, for example, Peacocke & Schachman (1954) and Johnson, Shooter & Rideal (1950).

$S_{20,w}$ values. These are approximately in the ratio 1:1·4:1·8. Two interpretations of these figures may be made on the basis of models representing extremes in shape. Thus if 'urease monomer' (the $S^0_{20,w}$ 20 component) is considered to be spherical and the dimer and trimer to be cylinders of axial ratio 2 and 3 respectively, it may be shown

on the basis of elementary theory and the equations of Perrin (1936) (tabulated by Svedberg & Pedersen, 1940) that the three sedimentation coefficients would lie in the ratio $1:1{\cdot}5_2:1{\cdot}8_7$. These differ little from the values for spheres quoted by Kegeles & Rao (1958). The alternative interpretation is based on very similar reasoning but correlates the observed frictional-coefficient ratio of 1·19 [Sumner *et al.* (1938) and this work] with asymmetry alone. By this means one obtains about 4 for the axial ratio of the 'monomer'. If the polymers are taken to have axial ratios of 8 and 12, the corresponding values of the sedimentation coefficients lie in the ratio $1:1{\cdot}3_1:1{\cdot}5_4$. Although both of these calculations must be considered as approximate, the similarities in the predicted ratios with those observed lends considerable support to the initial hypothesis. On some models, the $S_{20} = 36$ component could correspond to a tetrameric unit.

Electrophoretic-mobility values. Calculations here are somewhat more uncertain owing to the approximations involved in the theory, but applying the equations referred to by Abramson *et al.* (1942) to the case of a spherical monomer and cylindrical dimers and trimers, as before, and assuming that the total charge is conserved, one obtains mobility values in the ratio $1:1{\cdot}0_9:1{\cdot}1_2$. The second model described (cylinders of axial ratio 4, 8 and 12) gave lower mobilities, but these lay in the same ratio as for the first model. This result is partly coincidence, and does not imply that the mobility ratios are independent of the geometry of the model; thus a change from spherical monomer to dimer of axial ratio 4 produces mobilities in the ratio $1:0{\cdot}8_9$. In short, the theory predicts relatively small changes in mobility as a consequence of aggregation, and that these could either increase or decrease the mobility relative to that of the monomer, depending on the model used.

To sum up, it is clear that if the aggregation process is visualized as taking place between nearly spherical monomer units and that if the essential structural integrity of the monomer is largely or wholly preserved in the dimers and trimers (or tetramers), then this picture is compatible with both the ultracentrifuge and electrophoresis results. Analogous behaviour has been recorded for several other systems, e.g. L-amino acid oxidase (Blanchard, Green, Nocito, Ratner & Moore, 1945), haemocyanin (Svedberg, 1937), ground-nut globulins (Johnson *et al.* 1950; Johnson & Naismith, 1953), α-keratose (O'Donnell & Woods, 1956) and soya-bean proteins (Briggs & Wolf, 1957).

Effect of sulphite. The apparent conversion of heavier material into one of $S^0_{20,w}$ 20, caused by this reagent, is most directly explained on the assumption that the dimers, trimers etc. arise by the formation of intermolecular disulphide linkages; this linkage is known to be cleaved in mild and specific fashion by sulphite ions at pH 7. Although bisulphite ions must be present to a minor extent at this pH (Ambrose *et al.* 1950), it is not thought that bisulphite is concerned in the postulated disulphide-bond cleavage, in view of its relative inefficiency (compared with sulphite) in this respect (Cecil & McPhee, 1955*a*, *b*; Parker & Kharasch, 1959). Clearly, however, the action of bisulphite may be important at lower pH values, or generally as an inhibitor (cf. Ambrose *et al.* 1950).

The increase in area of the $S^0_{20,w}$ 20 component cannot always be correlated quantitatively with the disappearance of faster components. This might be due to neglect of material of very high molecular weight, evident at the bottom of the cell, which may also be affected by the sulphite. The most probable interpretation is that the species present in sulphite solutions has the same molecular weight as the $S^0_{20,w}$ 20 component in untreated solutions; this, if substantiated, has the consequence that either there can be no structurally important disulphide linkages in urease, or that, if they are present, they are considerably less 'accessible' than the intermolecular linkages.

Two points of interest in connexion with the polymerization remain. These are (*a*) whether it is an equilibrium reaction (in the sense that comparable proportions of e.g. monomer and dimer can exist at equilibrium at a realizable concentration) and (*b*) the related question of the mechanism. Concerning point (*a*), the effects of standing, of dilution and of temperature variation all indicate that, if an equilibrium exists, the kinetic constants regulating the interconversion are rather small. This conclusion is supported by the appearance of the centrifuge diagrams, which show clearly that the concentration gradient falls to zero between the peaks, thereby implying independence of the flow of each component (Gilbert, 1959; Williams, Van Holde, Baldwin & Fujita, 1958). This behaviour may be compared with that of such systems as α-chymotrypsin (Massey, Harrington & Hartley, 1955) and β-lactoglobulin (Ogston & Tilley, 1955), where rapid equilibration occurs between monomer and polymers. In respect of (*b*), the mechanism of polymerization, intermolecular disulphide bridges could arise either by direct oxidation of sulphydryl groups on separate molecules or by the mercaptan–disulphide interchange, originally proposed by Huggins *et al.* (1951) to account for the gelation phenomena encountered with plasma albumin in urea solutions. The evidence presently available does not allow a choice to be made between these possibilities and further discussion on this point will be deferred.

Urease solutions containing sulphite are colourless and relatively stable, and accordingly more

confidence can be placed in the quite reproducible physicochemical data obtained with them. Nevertheless, the results do not allow a reliable value of the molecular weight to be derived. The extrapolated sedimentation coefficient (in sulphite) $S^0_{20,w}$ could be combined with a similarly extrapolated $\mathscr{D}_A$ if such a value were available, or combination of S and $\mathscr{D}_A$ at the same finite concentration could be attempted. The latter procedure, which is normally a good practice, when there are grounds for assuming that $\mathscr{D}_A$ approximates to D_{11}, is not justified in this case because (*a*) the concentration dependence of S is rather pronounced, (*b*) even more doubts than usual must arise about whether an expression valid for two components can justifiably be applied to the system urease–buffer–sulphite–water, (*c*) $\mathscr{D}_A$ must differ from D_{11} by an amount at present unknown, but certainly significant. Confirmation of the presently accepted value of the molecular weight is evidently desirable.

SUMMARY

1. Electrophoretic-mobility data on urease have been obtained over the range pH 3·5–9·0; by interpolation it is confirmed that the isoelectric point of the enzyme in 0·10 ionic strength acetate buffers is 5·0 ± 0·05. The material gave a single, rather symmetrical peak at all pH values.

2. Sedimentation-velocity experiments generally gave at least three peaks, the relative proportions varying somewhat with the particular sample: the S_{20} values were approximately 19, 28 and 36 s.

3. In the presence of sulphite, samples which previously gave three peaks gave only the one with $S_{20} \sim 19$, and the specific enzymic activity increased. The concentration-dependence of the S of this species has been determined.

4. Diffusion experiments gave much lower values of $\mathscr{D}_A$ in the absence of sulphite than in its presence, and the deviations from Gaussian form of the diffusion curves also changed markedly.

5. The data are interpreted as indicating that dimers and trimers tend to be formed in urease solutions, but that the system is not one in rapid equilibrium: the polymers are probably formed by intermolecular disulphide cross-linkages.

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Carbohydrates in Protein

2. THE HEXOSE, HEXOSAMINE, ACETYL AND AMIDE-NITROGEN CONTENT OF HEN'S-EGG ALBUMIN*

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One of us described more than 20 years ago (Neuberger, 1938) the isolation, from an enzymic hydrolysate of crystalline egg albumin, of a polysaccharide containing mannose, glucosamine and some unidentified nitrogenous material. Comparison of the hexose and hexosamine content of the protein and of the isolated polysaccharide together with the estimation of the molecular weight of the latter indicated that all the carbohydrate was present as one unit in the egg-albumin molecule. The analytical data obtained also suggested that, on the basis of the then accepted value of 40 000 for the molecular weight of the protein, egg albumin contains four residues of mannose and two of glucosamine. As the methods of isolation used involved treatment with acetic anhydride, no information could be obtained as to whether the glucosamine residue was present in the protein as its *N*-acetyl derivative. By the methods then available it was not possible to obtain any indication of the linkage between the carbohydrate and the peptide chain. Some revision of the data is also necessary in view of the fact that the molecular weight of egg albumin is now generally believed to be 45 000 (see Warner, 1954).

This work was taken up again some years ago and a short report of some new findings has appeared (Johansen, Marshall & Neuberger, 1958). Preliminary communications on the same subject from other Laboratories have also been recently published (Cunningham, Nuenke & Nuenke, 1957; Jevons, 1958). In the present paper the analytical methods used are critically examined and some of the earlier figures revised. Chromatographic evidence obtained with a hydrolysate of the polysaccharide, the preparation of which from egg albumin has been briefly described (Johansen *et al.* 1958), indicated that the only sugars present are mannose and glucosamine.

Mannose

Carbohydrate in proteins has generally been determined by reactions involving heating the material with conc. H_2SO_4 in the presence of an appropriate phenol, nitrogen base or related compound, giving rise to products, the intensities of the colours of which are measured. These methods have been applied to egg albumin. The orcinol–H_2SO_4 method as described by Winzler (1955) was used; but the extinction was read at a wavelength of 505 mμ, since it was found that, with a mannose standard, the spectrum of the coloured product had a steep curvature at a wavelength of 540 mμ but not at 505 mμ, and secondly that rectilinearity was achieved on plotting absorption against amount of mannose at 505 mμ but not at 540 mμ. A five-times recrystallized [$(NH_4)_2SO_4$] preparation

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of egg albumin (preparation M) was found to contain 1·93, 1·83 and 1·98, 1·88 % of mannose, and a five-times recrystallized (Na_2SO_4) sample (preparation P) 1·95, 1·96, 2·02 and 2·02 %. The spectra obtained with mannose, whole-egg albumin and the isolated polysaccharide (Johansen *et al.* 1958) were identical.

The orcinol–H_2SO_4 procedure as described by Hewitt (1937), in which the reagent concentrations and the time of heating are slightly different from those of Winzler (1955), was also applied. This method also gave about 2 % of mannose and the spectra obtained with mannose and egg albumin were identical. Neither of the methods appears to be influenced by the presence of amino acids or amino sugars when present in concentrations as high as are normally found in egg albumin. Previous results obtained have tended to be slightly lower than these values: 1·7 % by Sørensen (1934) and 1·8 % by Neuberger (1938) and Hewitt (1938).

With a solution of tryptophan-free purified glycopeptide, the estimation of mannose by the anthrone method, as described by Dische (1955), and by the Winzler orcinol method gave approximately the same value.

A problem which arises in connexion with the measurement of carbohydrate by such methods lies in ascertaining whether a solution containing a given quantity of a sugar will give a colour yield identical with one containing the same amount of sugar but combined in polysaccharide linkage. The work of Vasseur (1948) indicates that mannan after treatment for 20 min. with orcinol–H_2SO_4 reagent gives a spectrum 'which almost completely corresponds with that of mannose, and the dextran curve nearly parallels that of the glucose analogue'. The extinction of the polysaccharides, however, amounted to only about 90 % of those of the monosaccharides. It seems that values might tend to be a little low when obtained by such techniques.

The neutral-sugar content of hen's-egg albumin is thus probably five residues (2 %)/45 000 g. of protein, and that this sugar is largely mannose is indicated by (i) the identity of the spectra obtained with mannose and egg albumin in the orcinol–H_2SO_4 methods, and (ii) the preparation of the phenylhydrazone and the *p*-bromophenylhydrazone of mannose in about 70 % yield of that calculated from the orcinol value from a purified polysaccharide (Neuberger, 1938). Further support for such conclusions in the case of the polysaccharide is given when one considers the agreement obtained between the carbohydrate as estimated by the orcinol–H_2SO_4 methods (47–50 %) and the amount of fermentable sugar (56 %) in a 1·5N-HCl hydrolysate (Neuberger, 1938). However, confirmation of this conclusion by methods not based on colorimetry is desirable. Difficulties may still be encountered if the neutral sugars are combined with C-1 of hexosamine (Gottschalk & Ada, 1956).

Hexosamine

The estimation of the amino sugar content of proteins presents some special difficulties. The discussion below is centred largely on glucosamine, since egg albumin appears from the evidence given below to contain only this amino sugar. The principal methods available for the determination of amino sugars are the colorimetric methods of Elson & Morgan (1933) and its many modifications, that of Dische & Borenfreund (1950) and the deamination method due to Tracey (1952), as well as procedures involving isolation. All of these methods were applied to the amino sugars which have been liberated from the mucoprotein by acid hydrolysis and two problems arise in connexion with this: the hexosamine must be quantitatively liberated, and the possibility of its destruction must be considered.

Moggridge & Neuberger (1938) pointed out that substances such as α-methyl *N*-acetylglucosaminide can be cleaved under conditions of acid hydrolysis in two ways, as shown in Fig. 1. Hydrolysis may proceed either by cleavage of the glycosidic bond followed by that of the acetamido linkage (pathway I), or the reactions may occur in inverse order (pathway II), the former being the favourable pathway since it was shown that α- and β-methyl glucosaminide are extremely resistant to acid hydrolysis. Thus, for example, the rate of

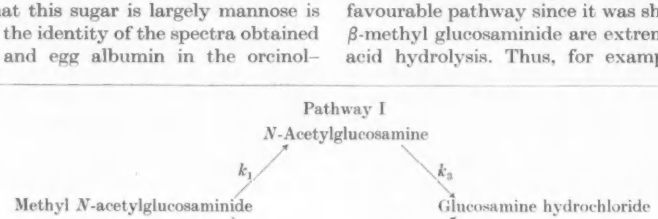

Fig. 1. Alternate pathways for hydrolysis of methyl *N*-acetylglucosaminide.

hydrolysis of the glycosidic linkage in α-methyl *N*-acetylglucosaminide in N-HCl at 100°, calculated from the results of Moggridge & Neuberger, is $1{\cdot}05 \times 10^{-3}$ hr.$^{-1}$, and the rate of hydrolysis of α-methyl glucosaminide under identical conditions is about $4{\cdot}05 \times 10^{-6}$ hr.$^{-1}$, the ratio of rates being of the order of 250 (Moggridge & Neuberger, 1938; Neuberger & Pitt-Rivers, 1939). The bearing of these results on the quantitative estimation of the amino sugar and neutral sugar content of mucoids and glycoproteins was appreciated at the time, but the problem was not clearly pointed out (see, however, Kent & Whitehouse, 1955) until the paper by Gottschalk & Ada (1956) appeared. Foster, Horton & Stacey (1957) made a study of the acid hydrolysis of a number of glucosamine derivatives and showed that glucosamine was not quantitatively liberated in general on hydrolysis in 1·16N-HCl at 100°. Any glucosamine combined in glycosidic linkage which was to be cleaved by the unfavourable pathway II would then be liberated only very slowly indeed, and possibly at such a rate that irreversible destruction balanced its rate of liberation. In order to ensure that the reaction should proceed by the more favourable pathway I, conditions of hydrolysis must be chosen so that the rate of hydrolysis of the glycosidic bond exceeds that of the acetamido bond as much as possible.

From the information available it would appear that the heat of activation of the glycosidic bond in α-methyl *N*-acetylglucosaminide is of the order of 36 000 cal./mole, while that of the acetamido bond is about 19 000 cal./mole (Moggridge & Neuberger, 1938). Although these values may vary from substance to substance it seems reasonable to assume that in general an increase in temperature will favour rupture of the glycosidic bond relative to that of the acetamido bond. Thus in N-HCl the ratio of the rate constants for glycoside to acetamido hydrolysis is 1·6 at 61·25° and 3·4 at 80°, so that a higher temperature would seem to favour the more desirable pathway I. The effect of acid concentration on the relative rates of hydrolysis (k_1/k_2) must also be considered. With methyl glucosaminide an increase in acid concentration from N- to 2·5N-HCl at 100° causes a nearly fivefold increase of rate constant (Moggridge & Neuberger, 1938). Such large increases in the rate of hydrolysis with increase in acid concentration have been observed in general for glycosides (Bunton, Lewis, Llewellyn & Vernon, 1955; Hantzsch & Weissberger, 1927). The amide-type bond shows much smaller increases in its rate of hydrolysis with acid concentration, however (Krieble & Holst, 1938). Thus conditions for obtaining the maximum yield of hexosamine from a glycoside would seem to involve the use of high acid concentrations and high temperatures, but the conditions must not be too vigorous since destruction of the amino sugar may occur.

We do not know the intrinsic stability of the glycosidic bonds in egg albumin and have therefore obtained values with various acid concentrations for different periods of time, hoping that the maximum value obtained approximates to the true glucosamine content of the protein. However, complete liberation of the glucosamine may not have occurred, some of the carbohydrate having undergone hydrolysis by the unfavourable pathway II. In such a case, disaccharides may have been formed which are almost completely resistant to acid hydrolysis, as in the hydrolysis of heparin. This might be particularly important if two *N*-acetylglucosamine residues were linked by 1:3 or, less likely, 1:1 (trehalose) linkages.

The glucosamine having been liberated, the most widely used method for its measurement is that due to Elson & Morgan (1933) or one of the numerous modifications thereof. This depends on the condensation of acetylacetone in mildly alkaline solution to give a pyrrole which Elson & Morgan thought to be 3-acetyl-2-methyl-5-(tetrahydroxybutyl)pyrrole. On reaction of the pyrrole with *p*-dimethylaminobenzaldehyde (Ehrlich's reagent) a red colour is produced and its intensity is measured. It was not until 1951 that Schloss (1951) showed that there were indeed at least two chromogens formed in the condensation of the amino sugar with acetylacetone, one of which was steam-volatile. This material condensed with Ehrlich's reagent to give a product absorbing maximally at a wavelength of 550 mμ. It was identified as 2-methylpyrrole by Cornforth & Firth (1958) and shown to be the major chromogen in the Elson–Morgan reaction. In the formation of such a pyrrole both the 3-acetyl and 5-tetrahydroxybutyl groups are eliminated, the latter by what is essentially a retroaldol fission involving C-3 of the original glucosamine molecule.

We were very fortunate in receiving in 1957 from Dr C. Cessi, then working at the Rowett Institute, Bucksburn, Aberdeen, details of a method for the quantitative estimation of hexosamine in which the steam-volatile chromogen noted by Schloss is distilled into the Ehrlich's reagent. The method is a modification of an earlier procedure (Cessi, 1952). The new procedure appears to decrease the interference, in the usual Elson–Morgan method, which has been observed when neutral sugars and amino acids are heated together (e.g. Horowitz, Ikawa & Fling, 1950; Vasseur & Immers, 1950; Sideris, Young & Kraus, 1938; Aminoff, Morgan & Watkins, 1952; Immers & Vasseur, 1952). The Cessi modification is also more specific than the original Elson–Morgan method in that it depends on the possibility of a

retroaldol fission occurring. Such a reaction is unlikely to take place with a 3-substituted amino sugar such as muramic acid or 3-methylglucosamine (Neuberger, 1941), and it has in fact been observed by us that these compounds do not give any colour in the Cessi modification of the Elson–Morgan procedure. Differences in the spectra obtained in the direct Elson–Morgan reaction with 3-substituted compounds and 2-amino sugars with a free hydroxyl group in the 3-position have been recorded. Cifonelli & Dorfman (1958) have shown that 3-methylglucosamine and hyalobiuronic acid exhibit maxima at a wavelength of 510 mμ, whereas glucosamine gives a maximum at about 535 mμ. Crumpton (1959) studied the reaction of muramic acid and demonstrated that there is not only a shift in the spectrum compared with that of glucosamine but also that the time of development of the full colour is delayed. It seems fairly clear that 2-methylpyrrole is not the chromogen produced in the case of 3-substituted hexosamines and it seems reasonable to suggest that it is 2-methyl-5-(2:3:4-trihydroxy-1-methoxybutyl)pyrrole where 3-methylglucosamine is involved. The Cessi procedure coupled with the original Elson–Morgan conditions could be used as a further diagnostic method in determining the possible presence of 3-substituted hexosamines.

The determination of the glucosamine content of two preparations of egg albumin, one a five-times crystallized [$(NH_4)_2SO_4$] sample and the other a five-times crystallized (Na_2SO_4) sample, was made. Hydrolysis of each preparation was carried out for 3 and 6 hr. in both 4N- and 5·7N-HCl. Glucosamine was estimated by the direct Elson–Morgan reaction as described by Rondle & Morgan (1955) and also by the Cessi modification. It is noteworthy that both the direct Elson–Morgan and the Cessi procedures give approximately the same results (Table 1). All the values obtained indicate that about three equivalents of glucosamine were liberated from both preparations of egg albumin and this is the minimum amount of the amino sugar that the protein contains. It is not impossible, as discussed above, that there are more than three residues of glucosamine in egg albumin.

The possibility existed that as well as the three moles of glucosamine, a 3-substituted hexosamine was also contained in egg albumin. A synthetic mixture was therefore prepared containing glucosamine and muramic acid in a molar ratio of 3:1, and a hexosamine assay carried out by the method of Rondle & Morgan (1955). The spectrum was observed after 5 min. and again after 18 hr. (cf. Crumpton, 1959). A hydrolysate of egg albumin (preparation M) was similarly examined. These results are shown in Fig. 2, where it is seen that by reading only on the 5 min. curve confusion might arise, yet after 18 hr. there is such a noticeable change in the spectrum of the material containing muramic acid that we may safely conclude that egg albumin does not contain this amino sugar and probably no other 3-substituted hexosamine stable to the hydrolysis under the conditions used.

Quantitative examination of the hexosamine content of a 4N-HCl hydrolysate (8 hr. at 100°) of the purified glycopeptide from egg albumin (Johansen *et al.* 1958), by the procedure of Moore & Stein (1951) as developed by Eastoe (1954), has been carried out. A sample of the glycopeptide containing 720 μg. of mannose was found to give 2·28 μmoles of an amino sugar in a position on the column corresponding to that of glucosamine, which is equivalent to 2·85 residues of glucosamine on the basis of 5 moles of mannose; there were also 1·55 moles of ammonia.

Table 1. *Glucosamine content of egg albumin after hydrolysis* (100°) *under various conditions*

Egg-albumin preparation	Concn. of HCl (N)	Time of hydrolysis (hr.)	Method of analysis	Glucosamine in protein (%)	Moles of glucosamine/ 45 000 g. of protein
$(NH_4)_2SO_4$-crystallized (preparation M)	4	3	Rondle & Morgan	1·13, 1·18	2·83, 2·95
	4	6	Rondle & Morgan	1·29, 1·23	3·21, 3·07
	5·7	3	Rondle & Morgan	1·35, 1·23	3·37, 3·07
	5·7	6	Rondle & Morgan	1·17, 1·18	2·93, 2·95
	4	3	Cessi	1·15, 1·12	2·89, 2·82
	4	6	Cessi	1·17, 1·17	2·94, 2·94
	5·7	3	Cessi	1·10, 1·15	2·77, 2·90
	5·7	6	Cessi	1·11, 1·10	2·89, 2·87
Na_2SO_4-crystallized (preparation P)	4	3	Rondle & Morgan	1·29, 1·28	3·21, 3·26
	4	6	Rondle & Morgan	1·15, 1·13	2·88, 2·83
	5·7	3	Rondle & Morgan	1·13, 1·26	2·83, 3·15
	5·7	6	Rondle & Morgan	1·07, 1·20	2·70, 3·00
	4	3	Cessi	1·10, 1·10	2·87, 2·87
	4	6	Cessi	1·22, 1·25	3·05, 3·14
	5·7	3	Cessi	1·21, 1·20	3·04, 3·02
	5·7	6	Cessi	1·17, 1·16	2·94, 2·92

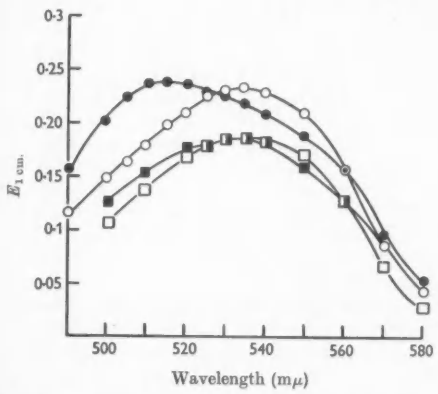

Fig. 2. Spectra given in the Rondle & Morgan (1955) determination of 2-amino sugars. Glucosamine (32 μg.) and muramic acid (3·28:1 molar ratio): ○, extinctions read after 5 min.; ●, extinctions read after 18 hr. Egg-albumin hydrolysate (4 N-HCl): □, after 5 min.; ■, after 18 hr.

Table 2. *Hexosamine content (Cessi procedure) of a purified glycopeptide from egg albumin after various conditions of acid hydrolysis* (100°)

Concn. of HCl (N)	Time of hydrolysis (hr.)	Residues of glucosamine/5 moles of mannose
2	3	2·49, 2·51, 2·52, 2·52
4	3	2·96, 2·98
4	6	3·02, 3·02
5·7	3	3·07, 3·07
5·7	6	3·01, 3·07

Hydrolysis of the purified egg-albumin glycopeptide was also carried out in 2 N- (3 hr.) and 4 N- and 5·7 N-HCl (3 and 6 hr.) and the amount of hexosamine liberated was measured by the Cessi procedure. The results of these experiments are given in Table 2, where the number of moles of glucosamine/5 moles of mannose are shown. The hydrolysis in 2 N-HCl appears to be less complete than those carried out in 4 N- and 5·7 N-HCl, results of a similar nature having been noted in connexion with the acid hydrolysis of ovomucoid (Marshall & Neuberger, 1960). The results again indicate that 3 moles of glucosamine/5 moles of mannose are liberated on acid hydrolysis.

Hewitt (1938) has reported a value of 0·8% of hexosamine in egg albumin after hydrolysis of the protein for 3 hr. in 2 N-HCl, whereas the results we have obtained under these conditions (Table 2) approximate to 1·0%. Sørensen (1938) has reported a value of 1·4% of hexosamine in the whole protein after hydrolysis in 5 N-HCl for periods of 3 and 6 hr., 0·54, 0·91, 1·13 and 1·22% after hydrolysis for 1, 3, 6 and 12 hr. respectively, in N-HCl and 1·25 and 1·19% after 2 and 3 hr. respectively in conc. HCl. Our results, we believe, demonstrate that there are certainly not less than three residues of glucosamine in egg albumin (1·2%), with the possibility of a fourth molecule.

During the analysis of the purified glycopeptide for its amino acid content by the method of Moore & Stein (1951) as described by Eastoe (1955), further data for glucosamine were also obtained. Hydrolytic conditions were rather more vigorous than those described above, namely 5·7 N-HCl at 115°; after 18 hr. hydrolysis values of 1·68 mole of glucosamine and, after 36 hr. hydrolysis, 0·79 mole of glucosamine/5 moles of mannose were obtained. Considerable destruction of glucosamine had occurred under these conditions, the degree being of a similar order of magnitude to that observed by Folkes, Grant & Jones (1950) when glucosamine was autoclaved for 5 hr. in 3 N-HCl in a sealed tube at 15 lb./in.² pressure (approx. 120°).

Amide nitrogen

Analytical data for the amide nitrogen of egg albumin have been reported on a number of occasions (Hausmann, 1899; Osborne & Harris, 1903; Pittom, 1914; Shore, Wilson & Stueck, 1935–36; Harington & Mead, 1936; Rees, 1946; Steven & Tristram, 1958). These determinations have all been carried out with acid medium for hydrolysis and are reported in Table 3. Alkaline conditions of hydrolysis have been used in one study (Warner & Cannan, 1942) and, although a result was obtained agreeing fairly well with that of Shore *et al.* (1935–36), such conditions are not recommended in view of the alkali lability of various amino acids.

Because of the instability of some of the amino acids under hot, strongly acid conditions, it would seem that the milder procedures should give the more accurate results. In particular, Rees (1946) showed that, under the hydrolytic conditions he employed, serine and threonine are deaminated only to a negligible extent.

All these earlier results are subject to error, however, for since that work was done it has become apparent that special precautions are necessary for determining the amide nitrogen content of proteins containing amino sugars. These are deaminated to a greater or lesser extent under the alkaline conditions generally used for the liberation of amide ammonia from a protein hydrolysate. Ammonia is quantitatively liberated from glucosamine and galactosamine provided that the conditions are rigorously standardized (Tracey, 1952).

In order, therefore, to determine the amide nitrogen in a hexosamine-containing protein, the following conditions must be observed: the hydrolysis of the protein must occur in such a way that

Table 3. *Amide-nitrogen content of egg albumin as reported by various workers*

Workers	Hydrolytic conditions	Amide N as % of protein
Hausmann (1899)	Conc. HCl. 5 hr. Boiling	1·28
Osborne & Harris (1903)	5·7 N-HCl. 7–10 hr. Boiling	1·35
Pittom (1914)	5·7 N-HCl. Time curve. Boiling	1·26
Shore, Wilson & Stueck (1935–36)	Time curves in 0·2 N-, N- and 5 N-HCl. 85° and 100°	0·97
Harington & Mead (1936)	5·7 N-HCl. 3 hr. 100°	1·06
	0·2 N-HCl. 20 hr. 100°	0·99
Rees (1946)	10 N-HCl. Time curve. 37°	1·04
Steven & Tristram (1958)	2 N-HCl. 3 hr. Boiling	1·04

the constituent amino acids are not deaminated; the protein hydrolysate must be steam-distilled under conditions whereby the hexosamine gives up its ammonia quantitatively without causing deamination of the amino acids; thirdly, care must be taken that the protein is not contaminated by free ammonia (Chibnall, Mangan & Rees, 1958). Since the result obtained will be a measure of the amide nitrogen plus the hexosamine nitrogen liberated, the latter must be subtracted from the value obtained to give the amide-nitrogen content. The amide nitrogen should also be directly measurable by precipitation of the ammonia in acid solution as its phosphotungstate, a method used by Berlin, Neuberger & Scott (1956), and this has been used in the present work.

Several preparations of egg albumin, details of which are given in the Experimental section, were used in this work. Hydrolyses of the various preparations were carried out with 10 N-HCl at 37° (Rees, 1946), and the amide and liberated glucosamine ammonia distilled by using the saturated sodium borate in saturated trisodium phosphate medium of Tracey (1952). In one experiment with protein preparation P, after periods of 18, 45, 96, 164, 216 and 264 hr., the apparent 'amide'-nitrogen values were 0·84, 0·96, 1·04, 1·07, 1·07 and 1·06% respectively. Similar time curves were obtained in the other preparations and gave asymptotic values for the 'amide'-nitrogen content of 1·04% for preparation M, 1·04–1·05% for preparation N, and 1·03–1·06% for preparation O. These final values indicate that the total amide nitrogen plus liberated hexosamine-nitrogen content of egg albumin lies between 33·1 and 34·4 residues per 45 000 g. of protein.

Glucosamine is either partially destroyed, or, more likely, incompletely liberated with the low temperature and high acidity used, as was indicated by following its rate of liberation from the protein by the Cessi procedure. After 24, 48, 96, 167, 217 and 266 hr. the apparent glucosamine content of the protein (preparation P) was 0·23, 0·34, 0·49, 0·64, 0·74 and 0·72% respectively. Results obtained after hyrolysis for 250 hr. with the other preparations are 0·70 (M), 0·73 (N), 0·72 (O) and 0·69% (P). The highest values correspond to slightly less than 2 equiv. of glucosamine/mole of protein.

The ammonia obtained on acid hydrolysis for 250 hr. under the same conditions, by precipitation as the phosphotungstate, was also measured. Values of 0·96 (M, 30·6 residues), 0·93 (N, 29·9), 0·99 (O, 31·9) and 0·97% (P, 31·2) were obtained. A similar method has been used previously for determining the content of the amide nitrogen in ovomucoid by Marshall & Neuberger (1960).

Our results indicate that in whole-egg albumin there are about 31 ± 1 residues of amide nitrogen, which is equivalent to about $0{\cdot}96 \pm 0{\cdot}03$% of amide nitrogen/100 g. of protein.

Acetyl content

Since in hexosamine-containing substances the amino groups of the amino sugars are almost always acetylated, an estimate of the amount of acetic acid produced on acid hydrolysis of both whole-egg albumin and the purified glycopeptide was carried out. The steam-volatile acid from a 2 N-HCl hydrolysate of the protein was distilled and subjected to column chromatography by the procedure of Bueding & Yale (1951). Yields of acetic acid under the conditions we used were of the order of 90%. Determinations of the acetic acid produced from egg-albumin hydrolysates gave values of 3·9 and 3·8 moles/45 000 g. of protein, allowing for a 90% recovery. After hydrolysis for 6 hr. the equivalent of 3·8 moles was produced.

Direct determinations of the amount of steam-volatile acid which were distilled from the (pH 3) hydrolysate were also carried out. The results obtained indicated values of 4·18, 4·15 (preparation M), 4·29 (N), 4·19 (O) and 4·00 (P) moles/45 000 g. of egg albumin. In all cases no acid other than acetic acid could be recognized by the gas–liquid partition chromatography method of James & Martin (1952).

Hydrolysis of the purified glycopeptide (3 hr., 2 N-HCl) gave rise to steam-volatile acid which was shown to be acetic acid (James & Martin, 1952), in amounts equivalent to 2·7 and 3·1 moles/5 moles of mannose. Thus the isolation of the glycopeptide

from whole-egg albumin results in a loss of one acetyl residue. It is possible that such a group is the substituent at the *N*-terminus of the protein chain, as egg albumin has no *N*-terminal amino acid when the usual dinitrophenyl techniques are used (Porter, 1950; Steven & Tristram, 1958). An *N*-terminal acetyl residue has been recognized for the tobacco mosaic virus protein (Narita, 1958*a*, *b*; Tsugita, 1960) and also in the α-melanocyte-stimulating hormone (Harris, 1959).

At least some of the three acetyl residues found in the glycopeptide are combined with the amino groups of the glucosamine residues, since acetyl glucosamine could be detected by paper chromatography after hydrolysis of the material in 0·5 N-HCl at 100° for 5 hr.

EXPERIMENTAL

Materials

Egg albumin. Preparations M, N and O were crystallized by the procedure of Sørensen & Høyrup (1915–17) as modified by Warner (1954). Recrystallization was carried out five times in all cases, followed by thorough dialysis at 4° against distilled water. One preparation was dried from the frozen state (M) and its moisture, ash and nitrogen contents determined (Chibnall, Rees & Williams, 1943). On a moisture-free, ash-free basis the nitrogen content of the protein was found to be 15·73%. A second preparation (N) was heat-denatured by the procedure of Chibnall *et al.* (1943), and a third (O) prepared by the same method and denatured by pouring an aqueous solution into cold methanol. Their nitrogen contents were 15·77 and 15·72% respectively. Preparation P was obtained by the method of Kekwick & Cannan (1936) whereby recrystallization was carried out five times with Na_2SO_4. The protein was dialysed and freeze-dried. Its nitrogen content was 15·66%. All these preparations were examined for the presence of free ammonia by the procedure of Chibnall *et al.* (1958) and found to be completely free from it.

A brief description of the isolation of the glycopeptide from egg albumin has been given by Johansen *et al.* (1958).

Methods

Carbohydrate estimations. These were carried out on the whole protein by the orcinol–H_2SO_4 methods of Winzler (1955) and Hewitt (1937), with mannose as a standard. Extinctions were read on a Unicam SP. 600 spectrophotometer. For the Winzler method the wavelength at which the extinction was read was 505 mμ. Orcinol from the British Drug Houses Ltd. was purified by sublimation. A protein solution (1 ml.) (1–1½%, w/v) was generally used. Amounts of glycopeptide were used containing similar levels of carbohydrate.

Glucosamine determinations. A sample of protein or purified glycopeptide solution (0·2 ml.) containing 30–40 μg. of glucosamine was placed in a tube fitted with a ground-glass stopper. The sample was dried *in vacuo* at room temperature over KOH and P_2O_5. A suitable dilution of constant-boiling HCl (1 ml.) was added, the stopper sealed in, and the solution heated in a boiling-water bath for the required time. The tube was cooled, the stopper removed and the contents were washed to the bottom of the tube. The solution was dried over KOH and P_2O_5 *in vacuo* at room temperature. The glucosamine content of the residue was determined either by the procedure of Rondle & Morgan (1955) or by the Cessi technique. The following description includes a slight modification which we have made to the method that Dr Cessi developed and kindly made available to us.

Cessi method for glucosamine analysis. The reagents used were: (i) acetylacetone reagent; 1 ml. of freshly distilled acetylacetone was dissolved in 25 ml. of M-Na_2CO_3 solution plus 20 ml. of water. The pH, where it differed from 9·8, was adjusted to that value, and the volume made up to 50 ml. This solution was never used more than 30 min. after it was prepared. (ii) Ehrlich's reagent; 2 g. of *p*-dimethylaminobenzaldehyde was dissolved in absolute ethanol, containing 3·5% of conc. HCl to a final volume of 250 ml. This solution was stored in the refrigerator.

In ground-glass stoppered tubes was placed 2 ml. of standard glucosamine hydrochloride solutions containing up to about 40 μg. of glucosamine (as the free base)./ml. Water (2 ml.) was used for the protein hydrolysates and for blank experiments. To each tube was added 5·5 ml. of acetylacetone reagent, and the tubes, with stoppers inserted, were heated together in a vigorously boiling water bath for 20 min. After being cooled in ice–water, the contents of each tube, in turn, were transferred to a 25 ml. flask together with 3 × 2 ml. of water washings. The flask was heated over a Bunsen flame and the steam-volatile chromogen distilled into a 10 ml. volumetric flask containing 8 ml. of Ehrlich reagent. Most of the chromogen came over in the first few drops of distillate. The extinctions were read after at least 30 min. at a wavelength of 548 mμ with a Unicam SP. 600 spectrophotometer in 1 cm. cells. Under these conditions 25 μg. of glucosamine (free base)/ml. gives an E 0·42, and the results are highly reproducible. Rectilinearity has been observed when a graph of E against μg. of glucosamine is plotted for values of between 4 and 40 μg. of amino sugar/ml. The colour values obtained are

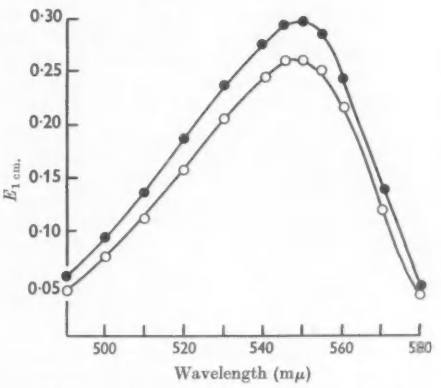

Fig. 3. Spectra given in the Cessi method for 2-amino sugars. ○, Glucosamine (46·4 μg.); ●, 4 N-HCl egg-albumin hydrolysate.

stable for at least 18 hr. The identity of the spectra obtained from glucosamine and from an egg-albumin hydrolysate is shown in Fig. 3.

Muramic acid and 3-methylglucosamine were subjected to a similar procedure at levels in each case of about 500 μg. of amino sugar/ml.

Precipitation of ammonia by phosphotungstic acid. A sample of hydrolysed protein (dried over KOH and P_2O_5 *in vacuo* at room temperature) in a centrifuge tube was dissolved in 5 ml. of water and 10 ml. of 30% (w/v) phosphotungstic acid which had been purified by the procedure of Van Slyke & Rieben (1944) was added. The pH was about 1. The tube was left at 4° overnight and centrifuged to separate the precipitate completely. The latter was washed twice by resuspending in 5 ml. of cold (4°) 30% phosphotungstic acid and centrifuging. The residue was steam-distilled with saturated Na_3PO_4 solution and the ammonia determined. In control experiments, with about 6–7 mg. of ammonia under these conditions, recoveries are of the order of 97–98%.

Acetyl estimations. The protein or the glycopeptide was hydrolysed in 2N-HCl, usually for 3 hr. The solution was cooled and neutralized to pH 3, and the steam-volatile acid distilled. The distillate was neutralized to phenolphthalein with NaOH and subjected to column chromatography by the method of Bueding & Yale (1951). In control experiments, sodium acetate gave rise to a yield of 86% of acetic acid, and acetic acid, when added to bovine-serum albumin and then hydrolysed, was recovered to the extent of 89%. The acetic acid level in such experiments was about 100 μmoles.

In the determinations in which direct measurement of steam-volatile acid was made, care was taken to ensure that CO_2 was excluded. The distillate was collected to a fixed volume (usually 25 ml.) and titrated against NaOH solution with phenolphthalein or thymolphthalein as indicator. Further fractions were collected and titrated until the titre value was equal to that of a similar volume of distillate from distilled water.

SUMMARY

1. Mannose is the only neutral sugar that has been recognized in egg albumin by paper chromatography. Colorimetric techniques have been applied and they indicate the presence in the protein of about 2% of this sugar, or five residues/45 000 g.

2. The difficulties which are liable to be encountered in liberating glucosamine from polysaccharide linkage have been discussed. The glucosamine liberated by hydrolysing egg albumin and the isolated polysaccharide from it under various conditions has been measured by both the direct Elson–Morgan reaction and also the Cessi modification of it. Both methods indicate a value of about 1·2% as the glucosamine content of the protein (three residues). The possibility of a fourth molecule of this or some other amino sugar cannot be completely excluded, for reasons which have been discussed. The Cessi procedure, which depends on the formation and steam-distillation of a steam-volatile chromogen (2-methylpyrrole), has been shown to be more specific than the original Elson–Morgan method. Thus 3-substituted glucosamines are not determined at all by this method.

3. Most of the recorded amide-nitrogen values of glycoproteins probably need correction, because 2-amino sugars are deaminated under the alkaline conditions used in the liberation of ammonia. This complication has been overcome by precipitating the ammonia with phosphotungstic acid. The amide nitrogen content of egg albumin is believed to be 31 ± 1 residues/mole.

4. The acetyl content of egg albumin has been shown to be four residues, and at least three of these have been shown to be associated with the carbohydrate moiety in the protein. The glucosamine is partly, if not wholly, *N*-acetylated. The possibility that the substituent at the *N*-terminus of the protein chain is an acetyl group has been discussed.

We wish to thank Dr C. Cessi, of the University of Bologna, Italy, who gave us details of his method for hexosamine analysis. Dr J. E. Eastoe of the Royal College of Surgeons kindly allowed us the use of his laboratory for the Moore & Stein analyses, as did Dr G. Popják of the Postgraduate Medical School, Hammersmith, in some of the early work on the fatty acid analysis by James–Martin chromatography. Dr M. J. Crumpton of the Microbiological Research Establishment, Porton, gave us a sample of muramic acid which he had prepared. Acknowledgement is made of the financial assistance given by the Medical Research Council to P.G.J. and by the British Empire Cancer Campaign to R.D.M.

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Some Factors Affecting the Permeability of Large-Granule Membranes to Mouse-Liver Catalase *in vitro*

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Approximately 70% of the catalase in mouse liver is present in the large granules, the remainder being in the extraparticulate cytoplasm (Adams & Burgess, 1957). Adams & Burgess (1959*a*) showed that when slices from mouse liver were incubated at 38° in a phosphate medium, catalase migrated from the granules to the extraparticulate cytoplasm. Also, when granules were isolated and incubated at 38° in 0·25 M-sucrose, catalase migrated into the surrounding medium. They produced evidence suggesting that no appreciable rupture of granules occurred under these conditions, and concluded that the migration was due to permeability of the intact granule membranes to catalase. In the same paper it was also shown that catalase migration was greatly reduced when slices in phosphate, or preparations of granules in sucrose, were incubated at 20°. No catalase migrated from granules prepared and incubated in phosphate medium at 38°. It appeared therefore that the granule membrane permeability depended greatly on the applied conditions, and the purpose of the present paper is to study these conditions further.

EXPERIMENTAL

Animals. Young adult mice of the CBA strain (Committee on Standardized Genetic Nomenclature for Mice, 1960) were used. The diet of the animals consisted of commercial rat cubes and water (both without restriction). The catalase migration from granules prepared from the livers

of these mice was qualitatively similar to that observed with granules from the Swiss albino strain used previously (Adams & Burgess, 1959*a*). However, the granules from CBA mice retained more of their catalase after incubation in 0·25 M-sucrose than did those from the albino strain.

Media used for liver homogenates. The phosphate medium used in some experiments was that described by Adams & Berry (1956), with the addition of 0·01 M-ethanol in order to prevent losses of catalase through the formation of an inactive complex of catalase with hydrogen peroxide ('Complex II', Chance, 1950; Adams & Burgess, 1959*b*). A similar addition of ethanol was made to all the media used. Except where otherwise stated, all homogenates and media were brought to pH 7·0 as previously described (Adams & Burgess, 1959*a*). Triton X-100 (0·25%) was added to liver homogenates or to large-granule suspensions when it was required to disrupt the granules and liberate their catalase into solution.

Preparation of catalase fractions from mouse liver. Livers were homogenized with 12 strokes of a Ten Broeck grinder, 1 g. of liver in 9 ml. of medium being used (Adams & Burgess, 1957). This will be referred to as a '12-stroke homogenate'. Various preparations were made from this. In some the homogenates were centrifuged at 800 *g* for 10 min. to remove nuclei and debris, the granules therefore remaining in contact with the soluble components of the liver. In others large granules were separated from the nuclei-free suspension by centrifuging at 10 000 *g* for 20 min. The granules were resuspended in the appropriate volume of fresh medium.

Catalase measurements were made at various times on the following preparations: (1) 12-stroke homogenate (i.e. extraparticulate cytoplasmic fraction and unbroken granules); (2) extraparticulate cytoplasmic fraction; (3) granule fraction (by addition of Triton to a granule suspension); (4) total catalase (by addition of Triton to a 12-stroke homogenate, or to one free of nuclei and debris); (5) supernatant catalase, i.e. catalase present in the supernatant after centrifuging resuspended granules. Except where otherwise stated, the homogenates or granule suspensions were diluted (1:10) with the appropriate medium before incubation. In some experiments where this procedure was not practicable (e.g. in the measurement of total protein released from granules), the concentrated homogenates were incubated. Where the first procedure was followed, sufficient tubes were incubated to enable a pair to be removed from the bath for each estimation. In the second procedure portions of concentrate were removed and diluted for the catalase estimations.

Estimation of liver-catalase activity. Catalase was estimated as previously described (Adams, 1950; Adams & Burgess, 1959*a*, *b*) and the activities of the various fractions were referred to the nitrogen content of the whole homogenate from which they were prepared. Nitrogen was estimated by the Kjeldahl method.

Measurement of oxygen uptake. Oxygen uptake by homogenates was estimated with conventional Warburg manometers and is given as mm.^{3} of O_2/hr./mg. dry wt. (q_{O_2}). The total volume of fluid in the flasks was 3 ml. The amount of homogenate or granule suspension added was 0·5 ml. (equivalent to about 50 mg. of whole liver), made up in 0·25 M-sucrose buffered with 0·02 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris), pH 7·4. Cofactors added in some experiments were (final concentrations): 25 mM-KCl, 1 mM-adenosine triphosphate (ATP) and 1 mM-sodium malate. The volume was made up with water containing 0·01 M-ethanol. The centre well contained 40% NaOH to absorb expired CO_2. The incubation temperature was 38°.

Polyvinylpyrrolidone. The sample had a molecular weight of 11 000.

Estimation of protein. Nitrogen was determined by the Kjeldahl method. In earlier experiments we expressed protein nitrogen as the difference between the total and non-protein nitrogen. The non-protein nitrogen was estimated in the supernatant after centrifuging the precipitate formed by the addition of an equal volume of saturated trichloroacetic acid. Since the values for the non-protein nitrogen were small and relatively constant, separate measurements of it were discontinued in later experiments, and the total nitrogen was taken as being equal to the protein nitrogen.

RESULTS

Effect of cations on the migration of catalase from large granules. A homogenate was made in 0·25 M-sucrose and freed of nuclei and debris. It was diluted immediately with various volumes of iso-osmotic solutions of calcium, magnesium, sodium or potassium chloride dissolved in water, and sufficient additional 0·25 M-sucrose was added to produce the standard 1:10 dilution. The resulting suspensions were incubated at 38° and estimations of catalase activity made at intervals until no

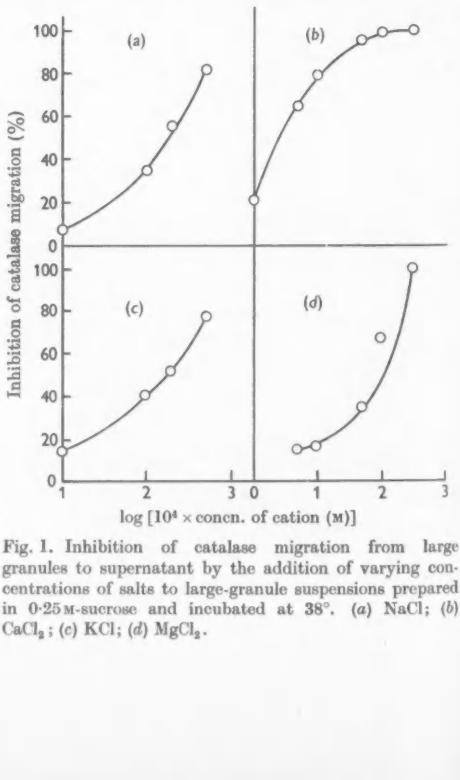

Fig. 1. Inhibition of catalase migration from large granules to supernatant by the addition of varying concentrations of salts to large-granule suspensions prepared in 0·25 M-sucrose and incubated at 38°. (*a*) NaCl; (*b*) $CaCl_2$; (*c*) KCl; (*d*) $MgCl_2$.

Table 1. *Rates of catalase and of protein release from granules during incubation of large-granule preparations in various media*

Granules were isolated and resuspended in the media indicated and incubated at 20° or 38° as shown. Catalase activities are given as arbitrary units/mg. of N. Protein values are in mg. of protein N/ml. of supernatant or of granule suspension.

Time of incubation (min.)	0			15			40		
(1) Granule suspension in 0·25 M-sucrose, incubated at 20°									
Supernatant catalase	89	74	60	84	81	80	95	81	78
Total catalase	294	364	283	335	364	283	335	356	272
Nitrogen in supernatant	0	0	0	0·16	0·17	0·21	0·18	0·17	0·19
Nitrogen in granules	0·51	0·59	0·45	0·36	0·37	0·25	0·36	0·33	0·28
(2) Granule suspension in 0·25 M-sucrose, incubated at 38°									
Supernatant catalase	89	76	60	154	138	114	221	186	174
Total catalase	294	387	264	350	378	248	350	365	252
Nitrogen in supernatant	0	0	0	0·26	0·19	0·20	0·25	0·24	0·22
Nitrogen in granules	0·51	0·59	0·40	0·34	0·37	0·21	0·29	0·35	0·19
(3) Granule suspension in phosphate medium, incubated at 38°									
Supernatant catalase	32	24	—	34	19	—	37	24	—
Total catalase	267	182	—	267	189	—	270	186	—
Nitrogen in supernatant	0	0	—	0·18	0·17	—	0·18	0·17	—
Nitrogen in granules	0·52	0·35	—	0·39	0·17	—	0·39	0·17	—

Table 2. *Catalase loss from granules prepared and resuspended in 0·25 M-sucrose and incubated for 2 hr. at 38° compared with that from granules centrifuged and again resuspended after 1 hr. incubation, and incubated for a further 1 hr.*

Catalase activities are expressed in arbitrary units/mg. of N.

Time of incubation (min.)	0	30	60	120
Supernatant catalase	38	172	165	170
Granule catalase	355	190	190	200
Total catalase	420	405	405	420

After 60 min. incubation samples were removed, the granules centrifuged and resuspended in 0·25 M-sucrose, and reincubated for a further 60 min.

Time of incubation (min.)	0	30	60
Supernatant catalase	7	11	14
Granule catalase	190	185	185
Total catalase	200	200	200

more migrated from the granules. This plateau was usually reached after 60 min. of incubation. The results (Fig. 1) show that all the cations inhibited catalase migration from the large granules and that calcium was the most effective. The total amount of catalase in the suspensions was unaltered during the incubation period. When the concentration of the bivalent cations was increased beyond 1 mM some coagulation of the granules occurred. In additional experiments sodium sulphate was substituted for sodium chloride. The inhibition curves were closely similar, and so are not separately reported.

Comparison of protein and catalase migrations from large granules suspended in various media. It seemed possible that the migration of catalase from the large granules into the surrounding medium in sucrose homogenates was part of a general protein migration. Granules were isolated and resuspended in the original media in which they were prepared, and the amount of protein lost from them into the supernatant during incubation for 40 min. was compared with the catalase loss. In these experiments (Table 1) total and supernatant catalase were measured.

Influence of extraparticulate-catalase concentration on catalase migration. Under our conditions between 30 and 70% of the granule catalase does not migrate on incubation. The following experiments were carried out to determine whether the rate of loss of catalase from the granules depends on the extraparticulate-catalase concentration. A homogenate was freed from nuclei and debris and the granules were centrifuged and resuspended in 0·25 M-sucrose. They were incubated at 38° for 120 min. Catalase migrated from the granules for the first 30 min. After 60 min. part of the suspension was centrifuged and resuspended in fresh sucrose. Practically no catalase migrated after this period from either the original preparation or from the resuspended granules (Table 2), showing that the cessation of migration is an intrinsic property of aging granules and is not dependent upon rise in concentration of extraparticulate catalase.

Influence of sucrose concentration in the medium on catalase migration. Livers were dissected from four animals, minced finely with scissors and carefully mixed together. The total was then divided approximately into four parts. The four samples were homogenized in 0·25 M-, 0·45 M-, 0·65 M- and 0·88 M-sucrose respectively, and samples of the resulting homogenates were incubated at 38°. The results appear in Fig. 2.

Effect on catalase migration of the addition of polyvinylpyrrolidone to 0·25 M-sucrose. Many attempts have been made to isolate mitochondria with minimum damage to the membranes. Among the methods used have been the addition of relatively inert substances of high molecular weight to the media, such as polyvinylpyrrolidone (Novikoff, 1956) and dextran (Birbeck & Reid, 1956). It seemed desirable therefore to examine the effects of such substances on our preparations.

The livers from four mice were prepared as in the last experiment, and homogenized in 0·25 M-sucrose and 0·25 M-sucrose made up to 2, 4 and 7 % (w/v) with respect to polyvinylpyrrolidone. Samples of the homogenates were incubated at 38° and the effect on catalase migration is illustrated in Fig. 3. Over the first 60 min. of incubation catalase migration progressively decreased as the concentration of polyvinylpyrrolidone increased.

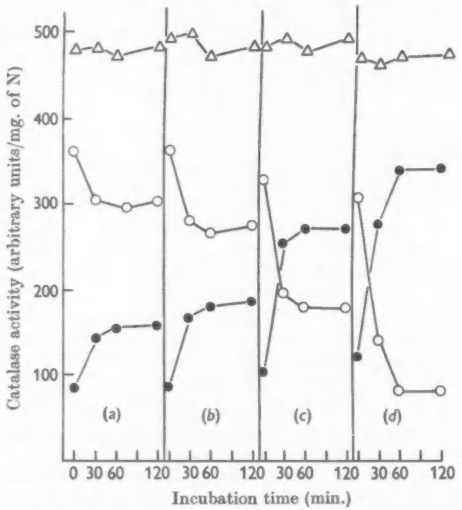

Fig. 2. Effect of sucrose concentration in the medium on catalase migration from large granules incubated at 38°. (*a*) 0·25 M; (*b*) 0·45 M; (*c*) 0·65 M; (*d*) 0·88 M. △, Total catalase; ○, granule catalase; ●, extraparticulate cytoplasm catalase.

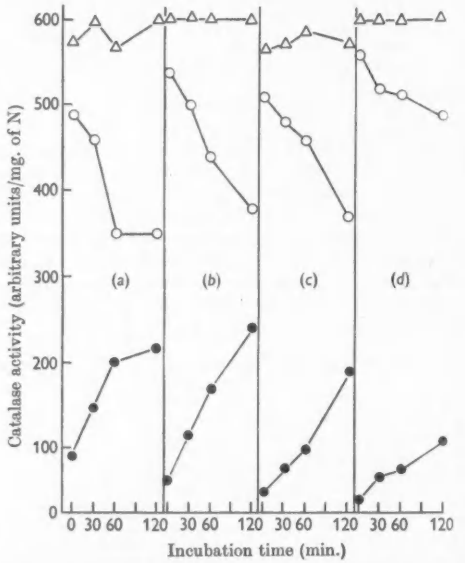

Fig. 3. Effect of the addition of polyvinylpyrrolidone to the 0·25 M-sucrose medium on catalase migration from large granules incubated at 38°. Concentrations of polyvinylpyrrolidone: (*a*) nil; (*b*) 2%; (*c*) 4%; (*d*) 7%. △, Total catalase; ○, granule catalase; ●, extraparticulate cytoplasm catalase.

Table 3. *Effect of the addition of respiratory cofactors (25 mM-potassium chloride, 1 mM-adenosine triphosphate and 1 mM-sodium malate) on catalase loss from isolated granules*

Granules were prepared and resuspended in 0·25 M-sucrose and incubated at 38°. Catalase activities are expressed in arbitrary units/mg. of N.

Time of incubation (min.) ...	0	30	60	90	q_{O_2}
No cofactors					
Supernatant catalase	35	200	225	222	4·0
Total catalase	370	375	380	390	—
With cofactors					
Supernatant catalase	42	140	190	195	22·3
Total catalase	350	375	400	370	—

Effect of addition of respiratory cofactors to large-granule suspensions on catalase migration. It was thought possible that the permeability of the large-granule membranes to catalase would be affected by the degree to which the granules were able to respire and metabolize. Liver homogenates prepared in 0·25 M-sucrose were incubated in Warburg vessels in the presence of sodium malate, ATP and KCl. These homogenates respired very much more rapidly than those to which no cofactors were added (Table 3), but the rate of appearance of catalase in the medium was only slightly decreased.

DISCUSSION

Adams & Burgess (1959*a*) concluded that catalase was capable of migrating through the intact membranes of large granules, and Adams (1960) suggested that this migration occurred *in vivo* as part of the mechanism regulating intracellular-catalase distribution. In view of the fact that catalase is a comparatively large molecule, it seemed desirable to seek further evidence that migration from large granules was not simply the result of damage to the membranes. In experiments with 0·25 M-sucrose as the basic medium, the addition of small concentrations of cations greatly decreased the catalase migration. The order of inhibitory activity was

$$Ca^{2+} > Mg^{2+} > Na^{+} = K^{+};$$

mM-Ca^{2+} ions produced 80% inhibition. On the other hand, increasing the sucrose concentration from 0·25 to 0·88 M progressively increased the rate of catalase migration. The addition of respiratory cofactors to 0·25 M-sucrose appeared to decrease catalase migration by only a slight amount. These results seem to confirm that the loss of catalase from large granules *in vitro* is a true migration of enzyme through the intact membranes and they show that permeability is greatly influenced by the composition of the surrounding medium.

There was a rapid protein loss from large granules during incubation, but the conditions under which this occurred differed markedly from those governing catalase loss. Thus the protein loss occurred during the first 15 min. of incubation, whereas considerable amounts of catalase migrated during the 15–40 min. period. Also, protein loss occurred in both phosphate medium and 0·25 M-sucrose incubated at 38°, and in 0·25 M-sucrose incubated at 20°. As previously found (Adams & Burgess, 1959*a*) no catalase migrated from granules incubated in phosphate medium, and very little from granules incubated in 0·25 M-sucrose at 20°. The results seem consistent with the supposition that protein was lost by elution from the granule surface.

The action of polyvinylpyrrolidone on the system (Fig. 3) was rather complex. For the first 60 min. of incubation the rate of catalase migration was progressively decreased as the concentration of polyvinylpyrrolidone in the medium increased. However, in the sucrose media studied, and also with the sucrose media to which cations had been added, catalase migration virtually ceased after 60 min. of incubation. When polyvinylpyrrolidone was present in the medium, the rate of catalase migration between 60 and 120 min. at all concentrations was equal to or greater than the rate from 0 to 60 min. It has always been difficult to explain why in plain sucrose media catalase should migrate steadily for 60 min. and then cease while a large amount is still present in the granules. The experiments given in this paper (e.g. Fig. 2) show that the proportion of catalase migrating differs considerably with the composition of the medium, and also is not influenced by the rise in extra-particulate-catalase concentration. In any event the cessation of catalase migration in this way would not be expected if the catalase release is due to membrane damage. Since it appears that granule membranes are in better condition in media containing polyvinylpyrrolidone, it also seems unlikely that the release of catalase during 60–120 min. of incubation in these media is due to membrane damage. Most probably the effect of polyvinylpyrrolidone is to slow the general rate of migration. In liver slices, for example (Adams & Burgess, 1959*a*), catalase migrates steadily from the granules during the 2 hr. of incubation, and more slowly from 2 to 4 hr. It was thought, however, that to incubate granules for more than 2 hr. would be very likely to produce serious membrane damage, which would render the interpretation of the results very difficult.

One other point of interest, shown in Fig. 3, is that the catalase activity present in the medium immediately after liver homogenization decreased steadily as the proportion of polyvinylpyrrolidone in the medium increased. About 90 catalase units were present in 0·25 M-sucrose, but only about 40 in 0·25 M-sucrose with 7% of polyvinylpyrrolidone. There seem two possible explanations for this. First, polyvinylpyrrolidone may assist in the preservation of structures present in the cytoplasmic reticulum, with the result that part of the extraparticulate catalase sediments with them. Or, secondly, there may be catalase-containing particles which are stable only in sucrose–polyvinylpyrrolidone solutions. In this case the extra-particulate catalase present in plain sucrose or phosphate media would contain some enzyme derived from particles. It is known (Thomson & Klipfel, 1958) that, in the rat, catalase activity is distributed over a range of different articles in the

large-granule fraction. However, increasing the sucrose concentration to 0·88M (Fig. 2) did not appear to decrease the catalase activity found in the extraparticulate fraction immediately after the preparation of the homogenate.

SUMMARY

1. The addition of cations to suspensions of liver large granules in 0·25M-sucrose decreases the catalase migration from them during subsequent incubation at 38°.

2. The addition of polyvinylpyrrolidone to such suspensions also decreases the catalase migration.

3. Catalase migration from granules is increased as the sucrose concentration in the medium is increased.

4. The addition of respiratory cofactors to granule preparations in 0·25M-sucrose has little or no effect on catalase migration.

5. When granules are incubated in either a phosphate medium or in 0·25M-sucrose, 30–50% of their protein rapidly appears in the medium. This is probably due to an elution of material from the granule surface, whereas the catalase loss appears to occur by migration through the granule membranes.

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Biochemical Changes in the Tissues of Animals Injected with Iron: Acid Phosphatase and other Enzymes

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Iron occupies a unique place among heavy metals, both in the large amount normally present in the body and in the special mechanism available for its storage in tissues as ferritin and haemosiderin. Syndromes associated with the storage of excessive quantities of iron are of clinical importance but have not been studied biochemically. The availability of iron for parenteral administration, in the form of an iron–dextran complex which displays low toxicity, makes it possible to increase by 100 times or more the total body iron of mice, rats and other animals. Apart from some reduction in growth rate there is no obvious adverse effect on the health and well-being of the animal, which can live its normal span (Golberg, Smith & Martin, 1957*a*). In the course of time, however, the rat which is rendered siderotic in this way develops many of the manifestations of vitamin E deficiency and there is strong evidence that the stored iron reacts with, or otherwise renders inadequate, the tissue reserves of antioxidant (Golberg, Smith & Martin, 1957*b*; Golberg & Smith, 1958). It has also been shown that the siderotic liver, although histologically intact, is particularly susceptible to toxic and adverse nutritional influences (Golberg & Smith, 1960).

Biochemical study of the grossly siderotic liver has revealed enhanced activities of some enzymes, with acid phosphatase showing an outstandingly large increase of an apparently permanent nature. An increase of this kind was observed (van Duijn, Willighagen & Meyer, 1958; van Duijn & Willighagen, 1959) for animals injected with polymers such as high-molecular-weight dextran or polyvinylpyrrolidone. With iron–dextran, enhanced liver acid-phosphatase activity appears to be attributable to excessive iron deposition, since moderate doses of iron, still well above the therapeutic doses in man, have no such effect.

METHODS

Iron dosage. Injections were given to albino mice, rats, guinea pigs and hamsters by the routes and in the doses indicated below. Two iron preparations were used: (*a*) iron–dextran complex (Imferon) containing 50 mg. of ferric iron/ml.; this stably chelated colloidal-iron preparation was the subject of British Patent Specification 748,024; (*b*) saccharated oxide of iron (Ferrivenin) containing 20 mg. of ferric iron/ml., which was mixed with an equal volume of horse serum (Evans Medical Supplies) to lower the pH and maintain the stability of the colloid. The low-molecular-weight dextran has a weight-average molecular weight of 6500–7600.

For organ analysis the animals were killed by stunning and exsanguination. The organs were quickly dissected out, washed to remove blood, blotted and weighed. They were homogenized in ice-cold water, in a cooled Potter–Elvehjem homogenizer fitted with a stainless-steel pestle which had been previously cooled to 0°. The homogenate was then macerated at room temperature without overheating, in a Nelco blender (MSE) for 3 min. to liberate all the enzyme activity (Wattiaux & de Duve, 1956). The volume of homogenate was adjusted to a tissue concentration of 20 mg./ml.

Liver analysis. For protein estimation 0·1–0·2 ml. of the homogenate was diluted with water to 5 ml. in a centrifuge tube, 1 ml. of 20% trichloroacetic acid was added and, after centrifuging at 1500 *g*, the precipitated protein was dissolved in 2 ml. of 15% sodium hydroxide with warming if necessary. Water was added until the volume was 9 ml. With tissues from iron-injected animals a precipitate of iron hydroxide was formed and was removed by centrifuging at 1500 *g*. To 8 ml. of the alkaline solution of the protein, 1 ml. of 5% copper sulphate was added. The suspension was kept for 10 min. with occasional mixing, shaken vigorously, centrifuged at 1500 *g* and the extinction of the supernatant was read at 555 mμ against a blank carried through the same procedure. A calibration curve was prepared from dried bovine albumin (fraction V, The Armour Laboratories). The results obtained by the method described were checked in selected cases by the Markham (1942) distillation method.

Ash was estimated by heating the tissue at 700–800° in a muffle furnace. Two or three drops of nitric acid were added to the ash, the crucible was reheated and the ash weighed. Moisture was determined by drying at 110°, and carbohydrate by the anthrone method (Bloom & Willcox, 1951). For nucleic acids the procedure of Schmidt & Thannhauser (1945) was used. After wet-ashing of the tissues with nitric acid and sulphuric acid, iron was determined colorimetrically (Ventura & Klopper, 1951).

Estimation of tissue-enzyme activities. The terms 'enzyme activity' and 'enzyme concentration' are applied to the results of various measurements, without prejudice to the question whether, when changes occurred, the amount of enzyme protein had varied or whether the activity of the enzyme/mg. of protein had altered.

Acid-phosphatase activity was measured in 0·2 ml. samples of tissue homogenate, with citrate buffer, pH 4·9, containing 0·01 M-disodium phenyl phosphate (King & Wootton, 1956*a*). For measuring alkaline-phosphatase activity at pH 10 the method of King & Wootton (1956*b*) was used with the same substrate. The unit of phosphatase activity was μg. of P released/100 mg. (wet wt.) of tissue/min. Since all experiments involved comparisons of normal and siderotic livers having substantially equal N concentrations, the specific activities of the enzymes measured were not calculated and all units are expressed per 100 mg. wet wt. of tissue.

For estimation of catalase activity the homogenate was diluted 100–200 times with 1% gum arabic; 1 ml. was taken for assay by the iodometric method, with hydrogen peroxide–phosphate buffer (pH 6·8) as the substrate (Sumner, 1941).

Catheptic and β-glucuronidase activities were estimated as described by Gianetto & de Duve (1955). For cathepsin, 0·39 mM-haemoglobin in 0·2 M-sodium acetate–acetic acid buffer, pH 3·6, was used, and the tyrosine released was measured according to Anson (1937). For β-glucuronidase the substrate was 1·5 mM-sodium phenolphthaleinglucuronide. With xanthine oxidase, 1 g. of liver was homogenized in 10 ml. of 0·1 M-sodium pyrophosphate buffer, pH 8·0, with a cold stainless-steel pestle and Potter–Elvehjem smooth-glass homogenizer. The homogenate was centrifuged at 1500 *g* at 3–6°. The supernatant layer was incubated at 37° for 1 hr., centrifuged again for 30 min. at 20 000 *g* at 3–6° and the clear supernatant layer was removed for assay. A portion (4 ml.) of 0·26 mM-xanthine solution in 0·1 M-buffer was placed in a temperature-controlled cell at 25°, 0·1 ml. of the supernatant was added and uric acid measured by the increase in extinction at 290 mμ at 1 min. intervals for 10 min.

The procedure described by Swanson (1955) was used for measuring glucose 6-phosphatase. Liver microsomes as a source of glucose 6-phosphatase were prepared by the method of Beaufay & de Duve (1954) and the concentration of microsomes was assessed by protein N. Adenosine triphosphatase was estimated in accordance with directions given by Kielley (1955), except that the final incubation was carried out at 38° instead of 28°. For non-specific esterase the procedure of Nachlas & Seligman (1949) was followed, with Fast Blue Salt B (Gurr). The method of Mahler, Sarkar, Vernon & Alberty (1952), as modified for crude tissue extracts, was applied to the estimation of reduced diphosphopyridine nucleotide (DPNH)–cytochrome *c* reductase. The arylsulphatase assays were performed according to Viala & Gianetto (1955). The manometric assay was used for uricase (Leone, 1955) because the brown colour of the siderotic-liver homogenates prevented the use of ultraviolet spectroscopy. The method of de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955) was used in determining acid deoxyribonuclease and ribonuclease; in the latter instance total phosphorus was measured rather than ultraviolet absorption.

RESULTS

Hepatomegaly

In the mouse, as in the rat and rabbit, a prompt and permanent increase in liver weight is an invariable response to heavy doses of parenteral iron. Histologically, the enlargement is seen to be due to a hyperplasia of all the liver elements. By chemical methods, comparisons have been made between the livers of untreated mice and those of mice given a series of daily intravenous injections of iron–

Table 1. *Composition of livers of untreated mice and of mice injected with iron*

Each value is the mean ± S.D. for a group of 10 male mice. The periods stated refer to the number of days after the last of nine daily intravenous injections of 0·1 ml. of iron–dextran.

	Mice injected with iron		Untreated mice	
	9 days	17 days	9 days	17 days
Body wt. (g.)	22·00 ± 4·14	26·25 ± 3·28	29·55 ± 2·73	37·40 ± 3·60
Liver wt. (g.)	1·96 ± 0·28	2·16 ± 0·39	1·37 ± 0·17	1·65 ± 0·26
Liver wt. (as % of body wt.)	9·00 ± 1·06	8·21 ± 1·08	4·64 ± 0·43	4·41 ± 0·42
Carbohydrate (mg./g.)	21·37 ± 8·31	6·79 ± 2·37	1·46 ± 0·77	3·27 ± 2·59
Protein (mg./g.)	—	224 ± 12·0	—	249 ± 15·4
Total solids (mg./g.)	—	269 ± 9·9	—	286 ± 5·2
Ash (mg./g.)	—	23·00 ± 0·82	—	21·10 ± 1·84

dextran. The liver content of water, ash, iron, carbohydrate, protein and nucleic acids has been measured. A few of the results are given in Tables 1 and 2.

The following generalizations may be made. There is stunting of growth in mice injected with heavy doses of iron, with an increase in the ratio of liver weight to body weight of 50–100% or more. On an average the liver contained 20–30% of the iron administered. Taking the groups overall, hepatomegaly in mice injected with large doses of iron is not reflected in differences of water or protein content. The additional ash content was the only constant feature. In animals killed at 9 days the carbohydrate content of the liver was 21·37 ± 8·31 mg./g. Included in this figure would be glycogen, residual dextran and possibly the carbohydrate component of haemosiderin. By 17 days the carbohydrate content of the siderotic livers had decreased to 6·79 ± 2·37 mg./g. This change may be accounted for by metabolism of the residual dextran.

A comparison of liver nucleic acids (Table 2) reveals that in the siderotic mice there is a decrease of ribonucleic acid P (RNA-P) and an increase of deoxyribonucleic acid P (DNA-P).

Liver acid-phosphatase activity

In mice the effects were first studied of factors such as age, sex, body weight and liver weight, as well as of multiple injections of 0·9% sodium chloride solution, low-molecular-weight dextran, clinical dextran and horse serum. Fig. 1 shows the relationship between liver weight and total liver acid-phosphatase activity in mice of different ages. In males the liver acid phosphatase increases moderately with increasing liver weight and age, but in old mice the liver phosphatase tends to fall. In females, increasing age and liver weight raise the enzyme level to a higher maximum than in the males.

In parallel experiments with groups of 10 male mice 6 weeks of age, the intravenous injection of iron, as iron–dextran complex or as saccharated oxide of iron, brought about striking increases in liver acid phosphatase (Table 3). Six hours after a single very heavy dose of iron there was a distinct increase in activity. Repeated injections progressively elevate the acid phosphatase, whether the iron is given as the dextran complex or as the saccharated oxide.

For a more accurate appraisal it is necessary to take into account the increase in liver weight which follows iron injections. A moderate excess of iron (5 mg. of iron/kg.), given daily, does induce hepatomegaly but any rise in acid phosphatase is small; given at weekly intervals, it increases neither the liver weight nor the enzyme activity. Twenty to forty times this dose produces a striking elevation of acid phosphatase (Table 4).

The untreated rat has a higher acid-phosphatase concentration per gram of liver than does the mouse. On injection of massive doses of iron the rise in acid phosphatase is less spectacular but still quite definite (Table 5). In the guinea pig the liver phosphatase remained unchanged. In both species anaphylactoid reactions were in evidence after multiple intraperitoneal injections of iron–dextran complex. Hamsters, however, tolerated the injections well and showed a sharp rise in liver acid phosphatase.

In Fig. 2 are shown some observations on the rate of increase in acid-phosphatase activity with time and the maintenance of the enhanced level over the ensuing weeks. After a single injection of saccharated oxide of iron the rise is more gradual than with iron–dextran. The high levels of acid phosphatase were maintained for 21 days but there was a suggestion that in time they might return to pretreatment levels. Accordingly, the long-term changes in liver phosphatase after massive doses of iron–dextran complex were studied in male and female mice given from 2 to 15 weekly subcutaneous injections and killed at various times after the last injection. Fig. 3 shows that even after only two injections of this kind a significant rise of liver phosphatase is present 6 months later. With 12 or 15 injections a large and persistent increase in enzyme activity has occurred.

Table 2. *Nucleic acids in livers of untreated mice and of mice injected with iron*

The periods stated refer to the number of days after the last daily intravenous injection.

Treatment	No. in group	Body wt. (g.)		Liver wt. (g.)		Liver nucleic acids: RNA-P (mg. of P/100 g.)		Liver nucleic acids: DNA-P (mg. of P/100 g.)		µg. of P/mg. of protein N		Ratio: RNA-P/DNA-P	
		Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.	RNA-P	DNA-P	Mean	S.D.
Untreated	10	22·7	1·5	1·08	0·10	80·3	6·8	23·6	2·4	22·5	6·61	3·42	0·23
Iron–dextran (3 × 0·1 ml.; 5 days)	6	23·2	3·1	1·59	0·77	57·2	8·0	35·7	4·1	16·4	10·2	1·66	0·24
Iron–dextran (3 × 0·2 ml.; 5 days)	10	22·9	3·4	1·68	0·70	59·6	6·2	34·9	3·7	17·9	10·50	1·72	0·21
Untreated	8	29·1	1·8	1·67	0·30	77·2	15·1	24·9	3·5	22·9	7·28	3·16	0·78
Iron–dextran (1 × 0·2 ml.; 1 day)	6	26·3	2·6	1·70	0·12	51·6	8·9	20·2	2·2	17·7	6·94	2·47	0·21
Iron–dextran (3 × 0·2 ml.; 1 day)	6	28·7	3·2	2·35	0·51	62·4	8·4	31·6	5·3	18·5	9·37	2·01	0·36

Fig. 1. Relationship between mean liver weight and total liver acid-phosphatase activity. ●, Groups of ten male mice; ○, groups of six female mice.

Acid-phosphatase activity in injected tissues

Repeated intramuscular or subcutaneous administration of iron–dextran in large doses leads to the accumulation of iron at the injection sites, as shown in Table 6. Two days after the last injection 25% of the total dose of iron given remained locally in the injected leg and 0·5% in the opposite leg; at 21 days there was 15% of residual iron. The thigh muscles and overlying skin and subcutaneous tissues showed a concomitant rise in acid-phosphatase concentration, rendered even more distinct than in the liver by the initial low values found in these tissues in the untreated animals. Two days after injection the iron content of the skin of the uninjected leg confirmed the naked-eye observation that there was an initial wide distribution of iron–dextran in the subcutaneous tissue throughout the body. By 21 days lymphatic clearance of the iron complex had occurred, and the residual iron was localized in macrophages as ferritin and haemosiderin (unpublished observations). The acid-phosphatase concentration of the tissue had increased by this time.

It was of interest to ascertain whether the rise in acid phosphatase at the injections sites is a permanent change, as it appears to be in heavily siderotic liver. The results in Table 6 show that this is so, in both mice and rats.

Acid-phosphatase activity of other organs

In view of the striking rise in liver acid phosphatase it was of interest to assess the corresponding enzyme levels in other organs of the mouse. Serum acid phosphatase was extremely variable and was apparently unaffected by the injection of large doses of iron. Significant changes were observed in the spleen, where high doses of iron–dextran complex, saccharated oxide of iron and

even low-molecular-weight dextran ($P < 0.01$) increased the acid phosphatase (Table 7). The 20% (w/v) dextran solution achieved this effect by increasing the size of the spleen from 156 ± 37.5 mg. in the group injected with 0.9% sodium chloride to 289 ± 99 mg. Saccharated iron oxide had a striking effect in one-quarter of the dose, as iron, as compared with iron–dextran. Again in the kidney, the saccharated oxide brought about a striking rise in acid phosphatase; iron–dextran in high dosage also raised the acid-phosphatase activity significantly ($P < 0.01$).

The results in Table 7 reveal some correlation between acid-phosphatase activity and the extent of iron deposition in these organs as judged histochemically by the method of Pearse (1953). When

Table 3. *Effect of treatment with iron on the acid-phosphatase activity of mouse liver*

Values are the mean results for 10 male mice. Injections were given intravenously.

Material injected	Dose per injection (ml.)	Dose per injection (mg. of Fe/kg.)	No. of daily injections	Time after last injection (hr.)	Acid-phosphatase activity (μg. of P/100 mg. of fresh liver/min.) Mean	S.D.
None	—	—	—	—	11·5	2·6
Sodium chloride (0·85%)	0·1	—	9	24	12·3	4·2
Low-molecular-weight dextran (20%, w/v)	0·1	—	9	24	12·4	3·3
Clinical dextran (6%, w/v)	0·1	—	9	24	11·4	2·7
Iron–dextran complex	—	450	1	6	25·2	4·6
Iron–dextran complex	—	220	1	24	24·7	7·0
Iron–dextran complex	—	220	2	24	33·9	5·1
Iron–dextran complex	—	220	3	24	39·8	3·9
Iron–dextran complex	—	220	9	24	58·0	13·4
Saccharated oxide of iron	—	182	9	24	50·3	11·6

Table 4. *Total acid-phosphatase activity of mouse livers*

Values are the mean results for 10 male mice, injected intravenously (i.v.) or intramuscularly (i.m.).

Material injected	Dose per injection (ml.)	Dose per injection (mg. of Fe/kg.)	No. of injections Daily i.v.	No. of injections Weekly i.m.	Liver wt. (g.) Mean	Liver wt. (g.) S.D.	Total liver acid-phosphatase activity (μg. of P/min./whole liver) Mean	S.D.
Sodium chloride (0·85%)	0·1	—	9	—	1·55	0·17	191	70
Low-molecular-weight dextran (20%, w/v)	0·002	—	9	—	1·60	0·43	169	56
Low-molecular-weight dextran (20%, w/v)	0·1	—	9	—	1·96	0·43	240	66
Iron–dextran complex	—	5	9	—	2·42	0·15	408	51
Iron–dextran complex	—	220	9	—	2·70	0·75	1532	139
Saccharated oxide of iron	—	90	10	—	2·06	0·34	1054	397
Saccharated oxide of iron	—	180	10	—	2·12	0·39	1015	269
Sodium chloride (0·85%)	0·002	—	—	10	2·38	0·32	216	26
Iron–dextran complex	—	5	—	10	2·57	0·32	237	35

Table 5. *Comparison of effects of treatment with iron in female rats, guinea pigs and hamsters*

Five daily intraperitoneal injections of 0·2 ml. of iron–dextran were given; animals were killed 48 hr. after last injection.

Species	Group	No. of animals	Mean body wt. (g.)	Mean liver wt. (g.)	Total liver acid-phosphatase activity (μg. of P/min.)
Rat	Untreated	10	158 ± 34	$6{\cdot}58 \pm 0{\cdot}75$	763 ± 94
Rat	Iron-treated	10	129 ± 14	$6{\cdot}70 \pm 0{\cdot}97$	1500 ± 388
Guinea pig	Untreated	5	283 ± 16	$13{\cdot}06 \pm 2{\cdot}14$	1267 ± 98
Guinea pig	Iron-treated	5	256 ± 25	$14{\cdot}64 \pm 2{\cdot}52$	1193 ± 204
Hamster	Untreated	5	97 ± 12	$4{\cdot}31 \pm 0{\cdot}86$	484 ± 181
Hamster	Iron-treated	5	107 ± 13	$5{\cdot}24 \pm 0{\cdot}47$	1104 ± 251

iron was given as the saccharated oxide, deposition in the kidney was greater than with 2·5 times the dose of iron–dextran. In a further experiment on the lines of group E, there were again clear-cut increases in acid-phosphatase activity of liver, kidney, spleen and small intestine. Heart, brain, lung, pancreas and testis were not significantly different in acid-phosphatase concentration from the untreated group of mice.

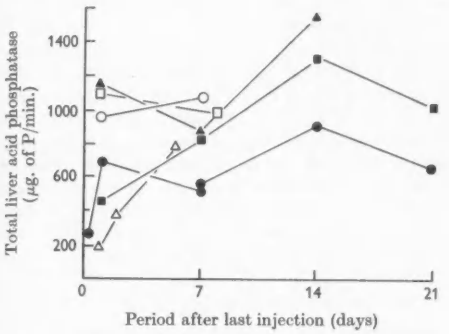

Fig. 2. Total liver acid-phosphatase activity of groups of ten male (M) or six female (F) mice at various times after the termination of daily intravenous injections of iron–dextran complex: ●, one injection of 0·1 ml. (F); ○, two injections of 0·1 ml. (M); ■, one injection of 0·2 ml. (F); □, three injections of 0·1 ml. (M); ▲, three injections of 0·2 ml. (M); △, one injection of 0·2 ml. of saccharated iron oxide (M).

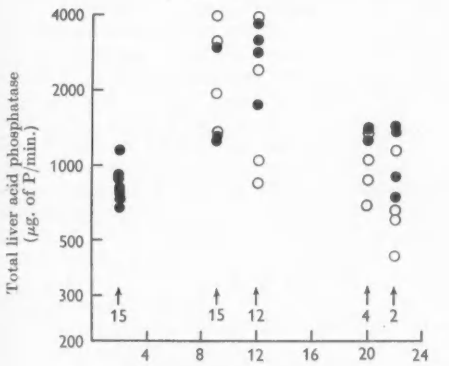

Fig. 3. Total liver acid-phosphatase activity in mice killed at various times after the termination of a series of weekly subcutaneous injections of 0·2 ml. of iron–dextran complex. The number of injections is shown under the arrow for each group. ●, Male mice; ○, female mice.

Table 6. *Effect of iron–dextran injections on acid-phosphatase activity at the injection site*

Results are expressed in terms of μg. of iron/100 mg. of fresh tissue and μg. of P/100 mg. of fresh tissue/min. Statistics refer to means ± S.D. Muscle refers to thigh muscles except in the mouse group marked *, where muscle from the lower anterior abdominal wall was used. In both groups marked * the data in the 'uninjected' columns refer to control untreated animals; in the two other groups injected with iron into one hind limb 'uninjected' tissues were derived from the other hind limb. 'Skin' includes subcutaneous tissues. Animals were injected intramuscularly (i.m.) or subcutaneously (s.c.).

Treatment	Species	No. in group	Period after last injection (days)	Uninjected				Injected			
				Muscle		'Skin'		Muscle		'Skin'	
				Iron	Phosphatase	Iron	Phosphatase	Iron	Phosphatase	Iron	Phosphatase
Untreated	Mouse	10	—	4·58 ±1·04	1·91 ±0·39	10·65 ±3·16	2·81 ±0·75	—	—	—	—
Iron–dextran (8 × 0·1 ml., i.m.)	Mouse	6	2	26·9 ±7·42	2·54 ±0·52	131·5 ±39·6	3·81 ±0·91	468 ±61·9	7·35 ±2·65	982 ±161	9·87 ±2·44
Iron–dextran (8 × 0·1 ml., i.m.)	Mouse	6	21	21·1 ±27·5	2·68 ±0·68	32·9 ±5·6	5·37 ±0·92	281 ±51·3	8·12 ±2·15	596 ±327	10·57 ±5·07
Iron–dextran (15 × 0·2 ml., s.c.)	Mouse*	6	400	—	1·91 ±0·38	—	2·10 ±1·00	—	12·76 ±4·45	—	15·87 ±8·73
Iron–dextran (22 × 75 mg. of iron/kg., i.m.)	Rat*	6	110	—	2·32 ±0·79	—	1·88 ±0·75	—	13·65 ±5·16	—	21·37 ±10·19

Table 7. *Acid-phosphatase activity of mouse organs*

Values are the mean results for groups of 10 male mice killed 24 hr. after the last injection. Group G is included here as a key to Fig. 4.

Group	Material injected	Dose			No. of daily intravenous injections	Acid-phosphatase activity (μg. of P/min.)							
						Kidney				Spleen			
		Vol. (ml.)	Dilution	(mg. of iron/kg.)		Activity/100 mg.		Total		Activity/100 mg.		Total	
						Mean	S.D.	Mean	S.D.	Mean	S.D.	Mean	S.D.
O	Untreated	—	—	—	—	7·7	1·6	28·0	4·5	26·2	3·8	52·0	27·4
A	Sodium chloride (0·85%)	0·1	—	—	9	10·1	1·9	38·7	6·8	46·6	6·8	72·4	19·6
B	Low-molecular-weight dextran (20%, w/v)	0·1	1:50	—	9	11·7	0·7	43·9	8·9	59·8	13·4	75·6	19·8
C	Low-molecular-weight dextran (20%, w/v)	0·1	—	—	9	11·0	0·6	45·9	7·7	46·8	12·6	127·3	35·1
D	Iron–dextran complex	0·1	1:50	5	9	9·7	2·1	45·4	5·9	46·6	10·3	90·9	18·9
E	Iron–dextran complex	0·1	—	250	9	18·8	4·0	57·6	10·1	100·8	35·1	194·7	51·3
F	Saccharated oxide of iron	0·1	1:1	100	10	28·4	8·1	91·1	20·5	104·2	28·4	270·6	51·1
G	Saccharated oxide of iron	0·2	1:1	200	10	—	—	—	—	—	—	—	—

Other enzymes in siderotic mouse liver

Three further enzymes were selected for detailed study in the liver of groups A–G (as listed in Table 7): alkaline phosphatase, β-glucuronidase and cathepsin. These activities are compared in Fig. 4 with the corresponding values for the sodium chloride-treated control group A. All three enzymes were significantly increased in group G, more particularly cathepsin, which showed enhanced levels in groups E and F as well. Moderate dosage with iron, as in group D, had no such effects.

Finally, groups A and E were selected for further study and a number of other enzymes likely to be affected by hepatic siderosis were measured (Table 8). Significant increases were found in arylsulphatase, acid ribonuclease, acid deoxyribonuclease and adenosine triphosphatase. The remainder were unchanged or reduced. Attempts were also made to measure hepatic cytochrome oxidase; these failed because the dark-brown colour of the blank, due to dissolved iron, rendered spectrophotometry impossible.

DISCUSSION

Liver enlargement and iron overdosage

The hepatomegaly which develops in mice after treatment with very large doses of iron–dextran or saccharated oxide of iron is a non-specific response to colloidal particles and macromolecules like colloidal carbon, thorotrast, dextran (low-molecular-weight and clinical types) and polyvinylpyrrolidone. It must be stressed that the doses used are high. With iron–dextran, the standard injection routine (9 × 0·1 ml.) introduces over 20 times the total body iron of the mouse and exceeds the total clinical dose (20 mg. of iron/kg.) by over 100 times. When smaller doses are given, still well in excess of the total body iron, no hepatomegaly can be detected. It is clear that an overloading effect is present and the analysis of liver RNA-P and DNA-P is suggestive of hyperplasia rather than hypertrophy. Thus in hypertrophied rabbit hearts Nowy, Frings & Rey (1959) found a fall in DNA-P, a rise in RNA-P and a rise in the RNA-P:DNA-P ratio from 2·9 to 4·0. In the siderotic mice, liver DNA-P rose and RNA-P fell, so that the ratio RNA-P:DNA-P fell from over 3 to 2 or less. The ratio reported by Schneider, Hogeboom & Ross (1950) for normal mouse-liver homogenate was 3·33; for rat liver Goodlad & Mills (1957) found a ratio of 4.

Significance of acid-phosphatase activity

To assess the importance of acid-phosphatase activity in siderotic liver it is necessary to consider the possible source of the increased activity.

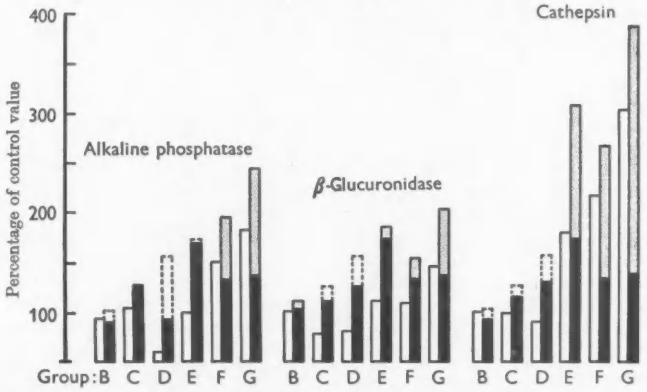

Fig. 4. Mean enzyme activities of mouse livers of groups B–G (for details see Table 7) in relation to those of the control group A (injected with 0·9% NaCl). Activities are compared on a 100 mg. basis in the left-hand columns and on a total liver basis in the right-hand columns. The ratio of mean liver weights for each group is marked on the right-hand column to indicate the extent to which the observed activity ratio exceeds (▤), or falls short of (□), what would be expected (■) merely from the mean changes in liver weight.

Table 8. *Enzyme contents of mouse livers*

Dosage of iron–dextran: five daily intravenous injections of 0·2 ml.; the mice were killed 24 hr. after the last injection. The groups marked *, † and ‡ received nine, three and two injections of 0·1 ml. respectively. Control groups received 0·9% NaCl in place of iron–dextran. Units are μmoles of substrate/100 mg. of liver/min., except in the following cases: uricase, μmoles of O_2; acid ribonuclease and deoxyribonuclease, E_{260}; DPNH–cytochrome *c* reductase, units defined by Mahler, Sarkar, Vernon & Alberty (1952). Statistics refer to means±standard deviations.

Enzyme	No. of mice in each group	Enzyme activity		*P*
		0·9% NaCl	Iron–dextran	
Arylsulphatase	10, 10	0·126±0·014	0·149±0·022	0·01–0·02
Acid ribonuclease	10, 10*	0·837±0·129	1·027±0·082	<0·001
Acid deoxyribonuclease	10, 10*	0·258±0·031	0·367±0·060	<0·001
Adenosine triphosphatase	6, 6	2·98 ±0·46	3·33 ±0·42	0·01–0·02
Uricase	6, 9*	0·395±0·050	0·402±0·088	>0·3
Xanthine oxidase	10, 10	0·047±0·027	0·032±0·016	>0·1
DPNH–cytochrome *c* reductase	5, 5	5·44 ±1·53	5·88 ±1·01	>0·6
Glucose 6-phosphatase	6, 6‡	1·26 ±0·23	0·89 ±0·15	0·001–0·01
	0, 6†	—	0·76 ±0·06	0·001–0·01
	10, 10*	1·51 ±0·36	1·21 ±0·49	>0·1
Catalase	10, 10	44·6 ±11·6	27·8 ±7·2	<0·001
Esterase	10, 10	16·1 ±3·6	10·9 ±2·5	0·001–0·01

Macrophage and related cell enzymes. On histochemical evidence Gedigk & Bontke (1957) have reported the presence of acid phosphatase, β-glucuronidase, phosphamidase, aminopeptidase and, less regularly, non-specific esterase; alkaline phosphatase and 5-nucleotidase were not present. Gropp & Hupe (1958) found that macrophages and epithelioid cells cultured *in vitro* gave positive histochemical tests for adenosine triphosphatase, non-specific esterase and acid but not alkaline phosphatase. It has been suggested by these and other workers (Weiss & Fawcett, 1953) that enzymic changes of this kind develop during the activation of resting histiocytic cells. Moreover, they appear to persist. The high enzyme activity of the foreign-body granuloma, for example, remains relatively constant over a long period and is only lost when fibrous scar tissue finally replaces the granuloma (Gedigk & Bontke, 1957).

The changes undergone by iron–dextran or saccharated iron oxide within splenic macrophages were studied by Richter (1959). He was able to

show by electron microscopy that within 6 hr. transformation to ferritin was taking place. Whereas saccharated iron oxide inhibits reticuloendothelial function for at least 48 hr., iron–dextran has a stimulatory effect (L. Golberg, L. E. Martin, C. Harrison & C. M. Bates, unpublished observations). It is likely therefore that under these conditions 'activation' and proliferation of liver histiocytic and Kupffer cells is taking place, and that this process is responsible for at least a part of the rise in acid phosphatase. The situation is clearer in injected muscle and subcutaneous tissues, where macrophage accumulation of striking proportions has been shown to take place within 48 hr. of massive iron–dextran injection (Beresford, Golberg & Smith, 1957; L. Golberg & S. Baker, unpublished observations).

Polymeric pigments. The presence of hydrolytic enzymes in lipofuscin or ceroid has been demonstrated by Gedigk & Bontke (1956) and Gedigk & Fischer (1959). Acid phosphatase is invariably found and non-specific esterase less constantly. The suggestion made by these authors, that lipofuscin is deposited at cytoplasmic sites of high acid-phosphate activity, is in keeping with the observation of Novikoff (1959) referred to below.

Parenchymal iron is always present in the liver of the siderotic rat or mouse and in the course of time its peribiliary location becomes more and more manifest. Histochemically demonstrable lipofuscin pigments develop only after many weeks (Golberg & Smith, 1960). However, the haemosiderin matrix which begins to form almost at once at the sites of iron deposition has many histochemical properties in common with ceroid (Weber, 1954), although Gedigk & Fischer (1958) have drawn attention to differences between them. The formation of lipofuscin around bile canaliculi led Pfuhl (1941) to suggest that the polymeric pigment develops in the Golgi body, a cytoplasmic structure which appears to be closely associated with, if not actually identical with, the 'acid phosphatase-rich granule' of Deane (1947). The first appearance of lipofuscin coincides with the disappearance of these peribiliary acid-phosphatase granules (Novikoff, 1959).

Although none of the above-mentioned histochemical studies includes comment on this point, it is difficult to decide whether haemosiderin shows a positive acid-phosphatase reaction. The Gomori (lead phosphate) technique fails, since, as would be expected, lead is fixed by the haemosiderin 'carrier substance' or matrix. The azo method (Pearse, 1953) reveals a distinct increase in acid phosphatase but is too diffuse to permit precise localization of enzyme activity. Haemosiderin granules when isolated by the technique of Ludewig (1957) show no acid-phosphatase activity *in vitro*.

Cytochondrial enzymes. Intense acid-phosphatase activity is the common feature of biochemical entities, the lysosomes, and cytological units: the Golgi bodies, 'acid-phosphatase granules' and 'peribiliary dense bodies' (Novikoff, 1959). In order to assess whether the increase of acid phosphatase is an index of generally enhanced lysosomal activity, other enzymes of this category were studied in siderotic liver: alkaline phosphatase, β-glucuronidase, cathepsin, arylsulphatase and uricase. Other phosphatases measured were adenosine triphosphatase and glucose 6-phosphatase, which is exclusively microsomal (Hess, Berthet, Berthet & de Duve, 1951), as well as esterase, said to be truly microsomal (Underhay, Holt, Beaufay & de Duve, 1956). Attempts to measure cytochrome oxidase as a typical mitochondrial enzyme were unsuccessful, owing to interference by iron as mentioned above.

Of the other enzymes measured, xanthine oxidase has been reported to be intimately linked with ferritin and with the liberation of ionic iron from its storage form (Mazur, Green, Saha & Carleton, 1958). DPNH–cytochrome *c* reductase was of interest in view of the possibility that α-tocopherol functions as the coenzyme. Hence inactivation of tocopherol, or its maintenance in the oxidized form, by the overwhelming accumulation of stored iron, might be expected to reduce drastically the observed activity of the enzyme.

The outcome of these measurements was to show that, in addition to acid phosphatase, a number of other hepatic enzymes had increased activity: alkaline phosphatase, β-glucuronidase and especially cathepsin; arylsulphatase, acid ribonuclease and deoxyribonuclease; adenosine triphosphatase. All of these except the last are lysosomal enzymes. Uricase remained unchanged. From the most recent evidence of de Duve (1959) uricase does not appear to run parallel with acid phosphatase. It may be present with catalase in some cytoplasmic particle other than the lysosome (Thomson & Klipfel, 1957). The fact that adenosine triphosphatase is not regarded as a lysosomal enzyme does not prevent the conclusion being drawn that injection of massive doses of iron exercises an effect on lysosomes and that the enhanced level of acid phosphatase is not entirely, though it may be partly, due to stimulation of reticuloendothelial-cell activity.

de Duve (1957) has criticized the use by Tsuboi (1952) of phenyl phosphate as substrate for the study of mouse-liver acid phosphatase at pH 5·5. This substrate is also split by glucose 6-phosphatase (Beaufay & de Duve, 1954), which is abundant in liver and has a pH optimum of 6·0. Tsuboi's high absolute values for acid phosphatase and low overall recoveries on fractionation of liver homogenate

are thus attributed by de Duve to this cause. In the present experiments carried out at pH 4·9 the contribution of glucose 6-phosphatase is slight. Direct measurements with glucose 6-phosphate as substrate at pH 6·5 (Table 8) showed either no rise or a distinct decrease in siderotic livers, whereas in the same livers there was a very large increase in acid phosphatase. Other experiments, to be reported separately, have involved the use of other substrates and various inhibitors, establishing beyond question that the enzyme involved is acid phosphatase and not glucose 6-phosphatase.

Role of iron

de Duve (1959) has reviewed the frequent, though not invariable, association of iron granules of high electron density with those cytoplasmic particles in liver, kidney and spleen which possess high acid-phosphatase activity. In earlier experiments, de Duve & Beaufay (1957) had demonstrated a highly significant correlation between the iron content and the acid-phosphatase activity of the lysosome-rich fractions of liver homogenate. Up to 10% of the cell ferritin was estimated to be associated with lysosomes or related particles, most of the remainder being in the final supernatant. More recently the same group (Beaufay, Bendall, Baudhuin & de Duve, 1959) have once more drawn attention to this correlation, suggesting that lysosomes and peribiliary dense bodies may be identical.

Bessis & Breton-Gorius (1959) have described iron accumulation within the mitochondria of sideroblasts and siderocytes, which rupture with the release of so-called '*micelles ferrugineuses*', supposedly a form of iron ready for incorporation into haem. Similarly, Richter (1958) has described particles containing much iron, the 'siderosomes', possibly derived from mitochondria. All these scattered observations suggest a relationship between iron in its storage forms and acid phosphatase in certain specialized cytoplasmic structures. In the present state of knowledge the functional connexion is obscure.

SUMMARY

1. Very high doses of iron, as iron–dextran or saccharated iron oxide, injected into mice, bring about stunting of growth and hyperplastic enlargement of the liver. There is a disproportionately large, and apparently permanent, increase in the acid-phosphatase activity of liver. Enhanced activity is seen in the liver of the rat and the hamster, but not of the guinea pig.

2. Injection of iron in doses which were less massive, yet well above the clinical level, or the administration of sodium chloride solution or dextran, had no such result.

3. In the mouse and rat the injection sites in muscle and in subcutaneous tissue have a raised acid-phosphatase activity after the administration of heavy doses of iron–dextran. The spleen and kidney of the mouse also show a higher level of acid phosphatase.

4. Of various other enzyme activities measured in the siderotic liver, the following were significantly raised: cathepsin, β-glucuronidase, alkaline phosphatase, arylsulphatase, acid ribonuclease, acid deoxyribonuclease and adenosine triphosphatase.

5. The significance of these observations is considered in the light of the possible relationships between intracellular iron and acid phosphatase on the one hand, and cytoplasmic particles or structures on the other. It is suggested that the observed changes may be partly lysosomal in origin but that the activity of macrophages and related cells is likely to be a contributory factor.

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Biochem. J. (1960) **77**, 262

The Inhibitory Action of Relaxing-Factor Preparations on the Myofibrillar Adenosine Triphosphatase

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Marsh (1952) reported that whole-muscle homogenates contained a factor (known as the Marsh factor, Marsh–Bendall factor or relaxing factor) which prevented adenosine triphosphate from inducing the normal synaeresis which occurred on addition of the nucleotide to washed muscle-cell fragments. Under certain conditions in the presence of the factor an elongation of the fibre fragments took place and the process was considered to be analogous to relaxation. In whole-muscle homogenates Marsh also observed that the adenosine-triphosphatase activity of the myofibrils was suppressed and subsequent work by others (Bendall, 1953; Bozler & Prince, 1953) showed that relaxation in muscle-model systems was associated with a low rate of hydrolysis of adenosine triphosphate. A number of investigators have demonstrated that so-called relaxing-factor preparations can also inhibit the Mg^{2+}-ion-activated adenosine triphosphatase of isolated myofibril preparations (Hasselbach & Weber, 1953; Portzehl, 1957*a*; Bendall, 1958).

The nature of this relaxing factor has been a matter of some controversy and although various phosphorylating systems (Bendall, 1954; Goodall & Szent-Györgyi, 1953; Lorand, 1953) have been claimed to possess activity it is now clear that particulate material can be sedimented from homogenates of rabbit skeletal muscle which will induce the relaxation of fibre models in the presence of adenosine triphosphate (Ebashi, Takeda, Otsuka & Kumagai, 1956; Portzehl, 1957*a*) and simultaneously inhibit the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase. These prepara-

tions do not require the presence of transphosphorylase systems (Portzehl, 1957*b*; Briggs, Kaldor & Gergely, 1959), although some workers (Molnar & Lorand, 1959) consider that such systems can potentiate their action.

Relaxing-factor preparations sedimented from muscle homogenates have been considered to be microsomal in nature (Molnar & Lorand, 1959), although the only evidence for this appears to be the fact that they require conditions for sedimentation which are comparable with those used to sediment the microsome fraction from other tissues.

This paper is concerned with an investigation of the distribution of the relaxing factor, assayed by its inhibitory action on the myofibrillar adenosine triphosphatase, in myofibril-free homogenates of rabbit and pigeon skeletal muscle. The properties of the preparation and its requirement for potentiating substances have been examined to throw light on the mechanism of inhibition of the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase.

METHODS

Preparation of granules of rabbit-skeletal muscle

Fractionation. Chilled minced back and leg muscles (30 g.) of the rabbit were homogenized for 2 min. at full speed in the MSE Ato-Mix blender (Measuring and Scientific Equipment Ltd.) in 300 ml. of either 0·25 M-sucrose or 0·08 M-KCl, each containing 0·039 M-borate buffer, pH 7·1 (Perry, 1953). After standing for 15 min. the homogenate was centrifuged at 2400 *g* for 25 min., the sediment of myofibrils and debris was discarded and the turbid supernatant centrifuged at 2400 *g* for a further 10 min. Three fractions were sedimented in succession from the myofibril-free supernatant as follows: (i) 30 min. at 8000 *g* (2400–8000 *g* fraction); (ii) 60 min. at 20 000 *g*, sometimes 23 000 *g* (8000–20 000 *g* or 8000–23 000 *g* fractions respectively); (iii) 60 min. at 90 000 *g* (20 000–90 000 *g* or 23 000–90 000 *g* fractions respectively).

In each case the sedimented material was carefully resuspended in a volume of medium equal to one-half of that of the original supernatant from which it was obtained, washed by resuspension in medium and sedimented by centrifuging at 15 000 *g* for 40 min. for fraction (i) and at 90 000 *g* for 60 min. for fractions (ii) and (iii). Finally the pellets were resuspended in one-twentieth of the volume of the original supernatant and stored at 0°.

'*Routine granule preparations*'. Minced back and leg muscles (120 g.) were homogenized with 300 ml. of 0·08 M-KCl–0·039 M-borate buffer, pH 7·1. Myofibrils and cell residues were removed by centrifuging at 2400 *g* for 25 min.; the supernatant was centrifuged for 60 min. at 20 000 *g*, the granules were washed by resuspension and centrifuging for 60 min. at 90 000 *g* and finally resuspended in medium of volume equal to one-sixth to one-eighth of the volume of supernatant from which they were obtained. All manipulations for granule preparations were carried out at 0°.

Preparation of granules of pigeon-breast muscle. Finely sliced pigeon-breast muscle (10–20 g.) was homogenized in the Potter homogenizer for 2 min. with 9 vol. of 0·25 M-sucrose–0·039 M-borate buffer, pH 7·1. The homogenate was centrifuged for 10 min. at 700 *g* to sediment myofibrils and cell residues. Some mitochondria were also sedimented by this treatment. The turbid supernatant was centrifuged at 8000, 23 000 and 90 000 *g* and fractions were prepared as described for rabbit muscle.

Preparation of myofibrils and muscle proteins. Myofibrils were prepared in 0·1 M-KCl–0·039 M-borate buffer as described by Perry & Corsi (1958). Natural actomyosin was extracted from isolated myofibrils and reprecipitated three times by the method of Perry & Corsi (1958). Synthetic actomyosin was prepared by combining (in equal proportions) myosin, prepared as described by Perry (1955), and F-actin, made by the method of Straub (1943).

Determinations of adenosine triphosphatase. Assays were carried out at 25° in a total volume of 2 ml. containing 40 mM-histidine–HCl buffer, pH 7·0, 5 mM-adenosine triphosphate (ATP; sodium salt), 5 mM-$MgCl_2$ and, unless otherwise indicated, 5 mM-dipotassium oxalate. When granules in 0·25 M-sucrose–0·039 M-borate buffer were used the assay medium was brought to a final concentration of 0·25 M with respect to sucrose. Tubes were pre-incubated and the reaction was started by the addition of 0·2 ml. each of granule and myofibril suspensions. Assays were carried out on myofibrils and granules incubated together and separately. Adjustment was made to ensure that the salt concentration was identical in each incubation and appropriate controls were also carried out. The reaction was stopped, usually after incubation for 5 min., by the addition of 1 ml. of 15% (w/v) trichloroacetic acid. Inorganic phosphate was estimated by the Fiske & Subbarow (1925) method with a 608 filter on the EEL colorimeter. The percentage inhibition of the myofibrillar adenosine triphosphatase was given by:

$$\frac{\text{Sum of inorganic P liberated by myofibrils and granules incubated independently} - \text{inorganic P liberated by myofibrils and granules incubated together}}{\text{inorganic P liberated by myofibrils alone}} \times 100.$$

In the oxalate systems the amounts of granules added were such that their intrinsic adenosine-triphosphatase activity was very low (< 5% of myofibrils). To compare the intrinsic adenosine-triphosphatase activity of the granular fractions, assays were carried out at different enzyme activities in the same medium as was used for the studies of inhibition of myofibrillar adenosine triphosphatase, to which 0·4 mM-dinitrophenol was added. All assays of the adenosine-triphosphatase-inhibitory activity of granules for fractionation studies in the absence of oxalate or other activator and of the adenosine-triphosphatase activity of the granules were carried out on the day of preparation. Assay of the adenosine-triphosphatase-inhibitory activity of rabbit-muscle granules in the presence of oxalate for fractionation studies was usually carried out on the day after preparation, as it was found that activity in the presence of oxalate did not alter significantly over this period. All estimations of pigeon-granule myofibrillar adenosine-triphosphatase-inhibitory activity were carried out on the day of preparation and in the presence of 5 mM-oxalate.

Succinic-dehydrogenase activity. This was measured manometrically as described by Slater (1949), immediately after preparation of the fractions.

Ribonucleic acid analyses. Determinations of ribonucleic acid content of granular fractions were carried out as described by Perry & Zydowo (1959). Total P and ribose estimations were made on the final hot 10% (w/v) perchloric acid extracts of the granules.

RESULTS

Distribution of relaxing-factor activity

Rabbit muscle. Investigations of the distribution of the activity of granule preparations in inhibiting the Mg^{2+}-ion-activated myofibrillar (adenosine-triphosphatase-inhibitory) activity were confined to studies on the turbid supernatants obtained when the myofibrillar fraction had been removed from cell-free homogenates of skeletal muscle. The results were essentially the same whether the homogenates were made in the KCl–borate or the sucrose–borate solutions (Fig. 1), although, in general, yields of granules were greater in KCl–borate solution.

Usually three fractions, so-called 2400–8000, 8000–20 000 and 20 000–90 000 *g*, were collected

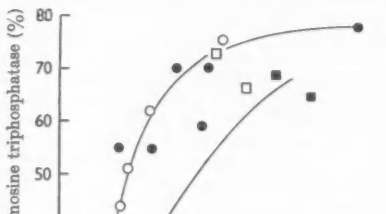

Fig. 1. Inhibitory activity of granular fractions sedimented from rabbit-muscle homogenates on rabbit myofibrillar adenosine triphosphatase. Assay conditions were as described in the Methods section. (1) 2400–8000 *g* fraction: ○, isolated in sucrose–borate; ●, isolated in KCl–borate; seven different preparations. (2) 8000–20 000 *g* fraction: □, isolated in sucrose–borate (at 23 000 *g*); ■, isolated in KCl–borate; four different preparations. (3) 20 000–90 000 *g* fraction: △, isolated in sucrose–borate; ▲, isolated in KCl–borate; four different preparations.

(see Methods section) and for a given fraction the specific inhibitory activity, i.e. the amount of granule nitrogen required to produce 50% inhibition of the myofibrillar adenosine triphosphatase, was remarkably constant and independent of the particular sample of muscle from which the fraction was obtained. If the percentage inhibition of the adenosine triphosphatase was plotted against granular nitrogen a response curve was obtained which was characteristic for each fraction (see Fig. 1). It will be seen that about 3·2 μg. of granule nitrogen gives 50% inhibition with the 2400–8000 *g* fraction, whereas about twice as much nitrogen is required for a similar response with the 8000–20 000 *g* fraction. The 20 000–90 000 *g* fraction was much less active on a nitrogen basis, and it was estimated that 90% of the total activity sedimented by the three centrifugings was obtained in the 2400–8000 and 8000–23 000 *g* fractions. The distribution of adenosine-triphosphatase-inhibitory activity was similar when measured with high concentrations of the fresh granules in the absence of 5 mM-potassium oxalate or with much lower concentrations in the presence of this substance (see section on effect of oxalate).

No clear separation in the distribution of succinic-dehydrogenase activity and adenosine-triphosphatase-inhibitory activity could be demonstrated between the three fractions. Although there was appreciable adenosine-triphosphatase-inhibitory activity in the lighter fractions, which were poorer in oxidative activity, the highest specific activity with respect to both properties was found in the 2400–8000 *g* fraction (Table 1).

In contrast with the above-mentioned properties no significant pattern of distribution of adenosine-triphosphatase activity or of ribonucleic acid content between the three fractions was apparent. Usually a total of about 0·34 mg. of nitrogen in the sucrose extracts, and 0·58 mg. of nitrogen in the KCl–borate extracts, per g. wet wt. of original muscle, was sedimented in the three fractions and distributed between them in a fairly constant manner (Table 1).

Pigeon-breast muscle. Homogenates of pigeon-breast muscle are very much richer in large granules than are similar preparations from rabbit skeletal muscle. These granules sedimented at relatively low speeds and to avoid too much loss in the preparation of the supernatants, myofibrils were removed by centrifuging in a lower centrifugal field than that used in similar preparations from rabbit muscle (see Methods section).

Granules of high specific activity for inhibition of rabbit myofibrillar adenosine triphosphatase were obtained in both the 8000–23 000 *g* fraction and the 23 000–90 000 *g* fraction. The most active pigeon

Table 1. *Inhibition of myofibrillar adenosine triphosphatase and other properties of granular fractions sedimented from homogenates of rabbit skeletal muscle*

Figures given are average values; numbers of experiments are shown in parentheses. Inhibition was measured under the usual assay conditions with about 0·2–0·6 mg. of myofibrillar N. For determination of succinic-dehydrogenase activity the three fractions prepared were: 2400–8000 *g*; 8000–20 000 *g*; 20 000–90 000 *g*. Assays of adenosine triphosphatase were done at 25°; succinic-dehydrogenase assays were done at 30°. Q_{O_2}: μl. of O_2/hr./mg. of N. Q_P: μl. of P/hr./mg. of N.

Homogenizing medium	Fraction	Distribution of total N (% of total sedimented)	Amount of granules to produce 50% inhibition (μg. of N)	Succinic-dehydrogenase activity (Q_{O_2})	Adenosine-triphosphatase activity (Q_P)	Ribonucleic acid content (mg. of ribonucleic acid P/g. of total N)
0·25 M-Sucrose, 0·039 M-Borate	2 400–8 000 *g*	20 (3)	3·2 (4)	21·8 (3)	2016 (1)	5·3 (3)
	8 000–23 000 *g*	50 (3)	5·5 (2)	3·8 (2)	1344 (1)	4·3 (3)
	23 000–90 000 *g*	30 (3)	20·0 (2)	0·9 (1)	2173 (1)	6·0 (3)
0·08 M-KCl, 0·039 M-Borate	2 400–8 000 *g*	21 (3)	3·5 (3)	—	—	—
	8 000–20 000 *g*	58 (3)	7·0 (2)	—	—	—
	20 000–90 000 *g*	21 (3)	85·0 (2)	—	—	—

Table 2. *Inhibition of myofibrillar adenosine triphosphatase and other properties of granular fractions sedimented from homogenates of pigeon-breast muscle*

Fractionation was carried out in 0·25 M-sucrose–0·039 M-borate buffer. Figures given are average values; numbers of experiments are shown in parentheses. Assays were carried out on fresh granule preparations under the same conditions as used for results shown in Table 1. Adenosine-triphosphatase assays were done at 25°; succinic-dehydrogenase assays were done at 30°. Q_{O_2} and Q_P are defined in Table 1.

Fraction	Distribution of total N (% of total sedimented)	Amount of granules to produce 50% inhibition (μg. of N)	Succinic-dehydrogenase activity (Q_{O_2})	Adenosine-triphosphatase activity (Q_P)
700–8 000 *g*	70 (5)	45 (2)	152 (2)	5420 (1)
8 000–23 000 *g*	—	20 (2)	—	—
	30* (5)	—	18* (2)	3810* (1)
23 000–90 000 *g*	—	20 (2)	—	—

* Estimated on fraction sedimented after centrifuging the supernatant, obtained after the 700–8000 *g* fraction was removed, for 60 min. at 90 000 *g*.

granules were, however, only about one-sixth as active on a nitrogen basis as the best preparations from rabbit tissue. In pigeon-breast-muscle preparations practically the whole of the succinic-dehydrogenase activity was sedimented in the 700–8000 *g* fraction. Although the specific adenosine-triphosphatase-inhibitory activity in this fraction was the lowest the total activity present was estimated to represent about half the total activity sedimented in all three fractions (see Table 2). Further, the range in specific activity between the fractions was less than with rabbit muscle.

Although the points made above clearly emerged, compared with those obtained with rabbit preparations the results with pigeon-breast muscle were much less consistent. In these studies pigeon-breast granules were assayed with rabbit myofibrils. This suggests that the effect is a general feature of muscle-granule and myofibril systems irrespective of their source.

Effect of oxalate

Relatively concentrated granular fractions isolated from rabbit muscle inhibited the adenosine-triphosphatase activity of myofibrils when the assay medium contained, in addition to buffer, only 5 mM-ATP and 5 mM-$MgCl_2$. It was observed, however, as reported by other workers, that certain substances had a marked potentiating action on the adenosine-triphosphatase-inhibitory activity. Oxalate, which was introduced into relaxing-factor systems by Portzehl (1957*a*), was particularly effective and at its optimum concentration (5 mM) the standard granule preparations (2400–20 000 *g* fraction) produced 50% inhibition of the myofibrillar adenosine triphosphatase at one-thirty-third of the nitrogen concentration required in its absence. Thus for 50% inhibition in the presence of oxalate under the normal assay conditions 7 μg. of 2400–20 000 *g* granule-fraction nitrogen was required, whereas for 50% inhibition in the absence of

oxalate, but under otherwise identical conditions, 230 μg. of nitrogen had to be present. Oxalate alone slightly depressed the adenosine-triphosphatase activity of myofibrils but for its potentiating effect to be apparent both granules and myofibrils were required to be present (Fig. 2). Oxalate could not be replaced by succinate, malonate, sulphite or hypophosphite. Citrate had some potentiating activity but was much less effective than oxalate. Pyrophosphate was comparable in effect to oxalate but at equivalent concentrations greater activity was always obtained in the presence of oxalate (Fig. 2). A further difference was that pyrophosphate had a greater depressing effect on the myofibrillar adenosine triphosphatase when this was measured in the absence of the granules. This was possibly due to the higher ionic strength of pyrophosphate solutions compared with oxalate solutions of equal concentration.

Low concentrations of calcium are well known to inactivate rabbit-muscle relaxing-factor preparations (Bozler, 1952; Bendall, 1953). Under the conditions employed in this work 0·1 mM-$CaCl_2$ completely removed the adenosine-triphosphatase-inhibitory activity of granule preparations used at concentrations of about 300 μg. of granule nitrogen-assay (Fig. 3), but this effect was prevented by the presence of 5 mM-potassium oxalate in the incubation medium. In the presence of 0·1 mM-$CaCl_2$ the intrinsic adenosine triphosphatase of the granules was increased, an effect which was likewise prevented by oxalate.

These results suggested that the action of oxalate could be explained by its property of combining with calcium and effectively removing it from the enzymic system. It was noticed in earlier experiments that often the inhibitory activity of granule preparations tested in the absence of oxalate fell off markedly on storage overnight at 0°. Similar changes in activity measured in the absence of oxalate occurred on incubation for 6 hr. at 35° in the presence of 2 mM-KCN. In both cases on assay in the presence of oxalate (at the same or at a very much lower nitrogen concentration) very little or no fall in activity could be detected (Table 3 and Fig. 4). Also associated with this apparent decrease in inhibitory activity was an increase in the endogenous adenosine-triphosphatase activity of the granules. The results were com-

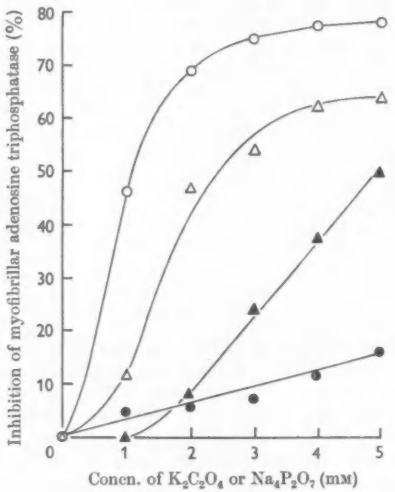

Fig. 2. Effect of dipotassium oxalate and tetrasodium pyrophosphate on: (*a*) Mg^{2+}-ion-activated adenosine triphosphatase of myofibrils; (*b*) inhibitory activity of rabbit-muscle granules on the myofibrillar adenosine triphosphatase. 'Routine granule preparation' (2400–20 000 *g* fraction) was used (29·3 μg. of N in the 0·2 ml. of suspension taken) in a total assay volume of 2 ml. Assay conditions were similar to those described in Fig. 1, except that varying concentrations of oxalate in pyrophosphate were present. ○, Myofibrils and granules incubated with oxalate; ●, myofibrils alone incubated with oxalate; △, myofibrils and granules incubated with pyrophosphate; ▲, myofibrils alone incubated with pyrophosphate.

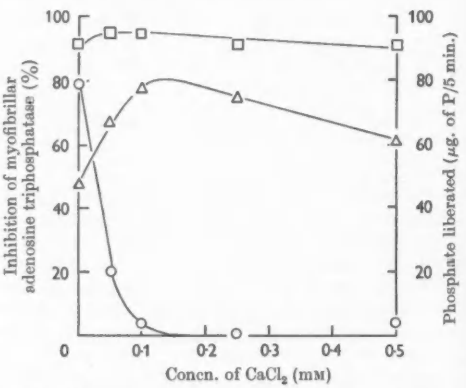

Fig. 3. Effect of $CaCl_2$ on the Mg^{2+}-ion-activated adenosine-triphosphatase activities of myofibrils and granules incubated independently and together. 'Routine granule preparation' (2400–20 000 *g* fraction) was used (336 μg. of N in the 0·2 ml. of suspension taken) in 2 ml. of assay medium. Assay conditions were similar to those of Fig. 1 except that oxalate was omitted and varying concentrations of $CaCl_2$ were added. □, Myofibrillar adenosine triphosphatase (expressed as μg. of P liberated); △, granule adenosine triphosphatase (expressed as μg. of P liberated); ○, adenosine triphosphatase of myofibrils and granules combined (expressed as percentage inhibition of myofibrillar adenosine triphosphatase).

Table 3. *Effect of aging and calcium chloride on the adenosine-triphosphatase-inhibitory action of rabbit-muscle-granule preparations*

Conditions were as described in the Methods section.

Granule preparation	Dilution used	Granular N used (μg./assay)	Conditions	Oxalate absent: Inhibition of myofibrillar adenosine triphosphatase by granules (%)	Oxalate absent: Intrinsic adenosine-triphosphatase activity of granules (μg. of P/5 min.)	5 mM-Oxalate present: Inhibition of myofibrillar adenosine triphosphatase by granules (%)	5 mM-Oxalate present: Intrinsic adenosine-triphosphatase activity of granules (μg. of P/5 min.)
A	Undiluted	—	Fresh	87	68·5	—	—
		—	Aged 8 days	20	212	88	37·5
A	50-fold	—	Fresh	—*	—*	64	3·2
		—	Aged 8 days	—*	—*	48	6·4
B	Undiluted	330	Fresh	79	60·5	89	56
		330	Aged 4 days	23	67·5	81·5	49
B	30-fold	11	Fresh	—*	—*	60	2·8
		11	Aged 4 days	—*	—*	59	2·4
C	Undiluted	—	Control	35	52	—	—
		—	6 hr. at 35°	15	82	91	42
C	35-fold	—	Control	—*	—*	64	1·6
		—	6 hr. at 35°	—*	—*	62	1·6
D	Undiluted	336	Control	79	48	—	—
		336	0·1 mM-$CaCl_2$ in assay	3	77	—	—
E	Undiluted	352	Control	70	76	90	72
		352	0·1 mM-$CaCl_2$ in assay	2	106·5	89	76

* Granule suspension was too dilute for assay in the absence of oxalate.

patible with the slow liberation from the granules on aging of some substance, possibly calcium, which destroyed the adenosine-triphosphatase-inhibitory activity and whose effect could be removed by oxalate.

When granules which had been stored for 3 days at 0° and then washed three times with KCl–borate buffer were assayed in the absence of oxalate they were found to possess greater adenosine-triphosphatase-inhibitory activity than an unwashed control sample of granules stored under identical conditions. In fact little loss of activity was observed in the repeatedly washed granule samples. Further, the inhibitory activity of granules dialysed against KCl–borate buffer containing 2 mM-KCN for 2 days fell off much less than an undialysed control sample stored in the presence of 2 mM-KCN. These results suggested that the decrease in inhibitory activity observed when granule preparations were allowed to age was due to the release of a dialysable substance which had the property of destroying the adenosine-triphosphatase-inhibitory activity of the granules.

An affinity of active granules for calcium was further suggested by the following experiment. Granules were suspended in KCl–borate buffer containing 10 mM-$CaCl_2$ for 1 hr. and then subsequently washed by repeated centrifuging and resuspension in KCl–borate buffer. On assay in the absence of oxalate after storage overnight the treated granules had considerably lower activity than did a control sample treated in an identical manner, except for the fact that the original KCl–borate buffer medium used for the initial dilution did not contain $CaCl_2$. In the presence of oxalate both samples had the same high inhibitory activity.

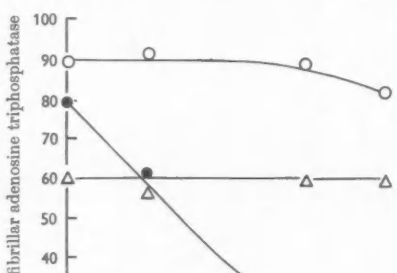

Fig. 4. Effect of storage at 0° on the adenosine-triphosphatase-inhibitory action of rabbit-muscle granules. 'Routine granule preparation' (2400–20 000 *g* fraction) was used (330 μg. of N in the 0·2 ml. of suspension taken, 11 μg. of N when diluted) in assay conditions identical to those of Fig. 1 except that oxalate was omitted in one series of experiments. ○, Myofibrils and concentrated granules with 5 mM-oxalate; ●, myofibrils and concentrated granules, oxalate omitted; △, myofibrils and diluted granules with 5 mM-oxalate.

Some properties of the granular preparation

It was not found possible to prepare an active acetone-dried powder, but freeze-dried granule suspensions retained activity after storage for several weeks.

By extraction with Weber's solution (0·06 M-KCl, 0·01 M-Na_2CO_3, 0·04 M-$NaHCO_3$) up to 25% of the total granular nitrogen was extracted but the adenosine-triphosphatase-inhibitory activity was unimpaired and remained associated with the insoluble residue. Treatment of a suspension of active freeze-dried granules with 0·3% of sodium deoxycholate resulted in destruction of the adenosine-triphosphatase-inhibitory activity. Subsequent centrifuging of the largely clarified suspension for 1 hr. at 15 000 *g* resulted in 67% of the total granule protein remaining in the clear supernatant. This supernatant, too, had no inhibitory activity. Similarly, treatment with 0·1 or 0·03% of deoxycholate did not yield active supernatants. The whole-granule suspension in 0·03% deoxycholate still retained activity, however. The removal of deoxycholate by dialysis did not alter these findings.

All granule preparations possessed adenosine-triphosphatase activity (see Tables 1 and 2), which was usually fully activated (Chappell & Perry, 1954) as very little stimulation was produced by 0·4 mM-dinitrophenol.

With a fixed amount of a granule preparation it was observed that the concentration of myofibrillar nitrogen could be increased by at least 200% without changing significantly the percentage inhibition of the myofibrillar adenosine triphosphatase, although the amount of hydrolysis of adenosine triphosphate ranged from 1·1 to 3·5 μmoles.

Inhibition was not affected by prior extraction of the actomyosin, for inhibition comparable with that obtained with myofibrils was obtained with natural and synthetic actomyosins (cf. Mueller, 1960). In these cases assays were carried out at somewhat lower ionic strength, as the Mg^{2+}-ion-activated adenosine triphosphatase of these systems was rather low in the usual incubation medium.

Neither Mg^{2+}-ion-activated myofibrillar inosine-triphosphatase activity nor uridine-triphosphatase activity was inhibited by granular preparations

Table 4. *Comparison of the inhibitory action of rabbit-muscle-granule preparation on the hydrolysis of adenosine triphosphate, inosine triphosphate and uridine triphosphate by myofibrils*

Incubation was carried out for 5 min. at 25° in a total volume of 2 ml. containing 0·2 ml. of granules or 0·2 ml. of myofibrils or both, 2·5 mM-$MgCl_2$, 5 mM-potassium oxalate, 40 mM-histidine–HCl buffer, pH 7·0, and 2·5 mM-nucleotide. ITP, Inosine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate.

Granule preparation	Granule N (used/assay) (μg.)	Substrate	Inhibition of myofibrillar nucleotidase by granules (%)	Myofibrillar-activity nucleotidase (μg. of P/5 min.)	Granular-activity nucleotidase (μg. of P/5 min.)
A (2400–90 000 *g*)	100	ITP	0	37	13·4
	100	ATP	79	90·5	14·8
	10	ITP	0	37	1·1
	10	ATP	36	90·5	2·1
B (2400–8000 *g*)	48	UTP	0	63	5·7
	48	ATP	76	84	8·5
	12	UTP	0	63	1 8
	12	ATP	41	84	2·2

which, under otherwise identical conditions, strongly inhibited the myofibrillar adenosine triphosphatase (Table 4).

DISCUSSION

Almost without exception all studies on relaxing-factor preparations reported in the literature have been confined to those isolated from rabbit skeletal muscle (cf. Bendall, 1954). In so far as the inhibitory action of muscle-granule preparations on the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase is an aspect of relaxing-factor activity, the present study provides evidence of the existence of a similar relaxing-factor system in pigeon-breast muscle. This is a highly oxidative tissue much richer in mitochondria than is rabbit skeletal muscle. In both tissues adenosine-triphosphatase-inhibitory activity was a property of all three of the granular fractions isolated although the specific activity was not identical in the fractions isolated from a given muscle type. There are suggestions that the structures with which the factor activity is associated are slightly different in pigeon-breast muscle compared with rabbit skeletal muscle. In homogenates of the former tissue material of highest specific activity was in the fractions containing slower-sedimenting components, whereas with the latter muscle the fraction with the faster-sedimenting components had the highest specific activity. In both rabbit and pigeon muscle the bulk of the oxidative activity and the major portion of the total sedimented-factor activity were associated with the heaviest-granule (mitochondrial) fraction.

With rabbit-muscle preparations at least it is clear that caution must be used in concluding that relaxing-factor preparations are comparable with the microsome fractions of other tissues. In rabbit muscle the fraction which sedimented under conditions which might be supposed to sediment microsomes, i.e. 23 000–90 000 *g* fraction, contained only 10% of the total sedimented relaxing-factor activity. Evidence has been provided by Perry & Zydowo (1959) that, unlike the microsome fraction of most other tissues, fractions isolated in a similar manner from muscle are not rich in ribonucleic acid, the bulk of which is sedimented with the myofibrillar fraction. Molnar & Lorand (1959) report that under their conditions they could detect little or no adenosine-triphosphatase-inhibitory activity in the material which was sedimented from 0·15M-KCl extracts in 30 min. at 11 000 *g*. In our hands a similar fraction, the 2400–8000 *g* fraction, assayed in the presence of oxalate was 25 times as active on a nitrogen basis as the so-called microsome fraction of Molnar & Lorand (1959) assayed in the presence of a potentiating phosphokinase system. Although the maximum yield of factor activity is associated with the mitochondria-rich fractions it does not follow that the factor is part of these structures, for active fractions with little or no succinic-dehydrogenase activity can be separated from both rabbit and pigeon muscle. This suggests that the normal muscle-mitochondrial fraction is heavily contaminated with elements containing the relaxing factor. Whatever these structures are, the factor activity is tightly associated with them. It is possible that the activity is associated with the reticular structures seen in electron-microscope studies of muscle (Porter & Palade, 1957) and which may play a role in conducting into the interior of the cell those changes taking place at the membrane which initiate contraction (see Huxley & Straub, 1958).

The questions of the role of dialysable cofactors (Briggs *et al.* 1959) or of transphosphorylase systems

(Molnar & Lorand, 1959) in relaxing-factor systems has not been investigated. Nevertheless, the observation that granule preparations on standing lose activity which can be restored by the addition of oxalate is possibly pertinent to these problems. Also it should be pointed out that the activity of factor preparations potentiated by cofactors from muscle extracts or by transphosphorylase is considerably less than the activity obtained in the presence of oxalate alone. The specific activity obtained for the heaviest fraction isolated from rabbit muscle when assayed in the presence of oxalate is the highest yet reported in the literature. It is significant that pyrophosphate, which behaves in a manner similar to oxalate, has many similarities in behaviour to the dialysable cofactor (Briggs *et al.* 1959). It could be that the cofactors act by decreasing in some way the effect of the inhibitory substance present in factor preparations which appears to increase in amount on standing and is rendered ineffective by oxalate.

The nature of this substance which is present in normal granule preparations and which is responsible for the decrease in activity on aging is not known. There is, however, strong presumptive evidence that it is calcium. Isolated heart sarcosomes possess a strong affinity for this cation and it has been shown that all the calcium originally present in the muscle may be concentrated in these structures on isolation. There is evidence, however, that the accumulation of calcium in the isolated sarcosomes may not necessarily indicate the distribution of this metal *in situ* (Slater & Cleland, 1953).

There are certain similarities between the properties of the preparations inhibitory to adenosine triphosphatase and oxidative-phosphorylating systems. In both cases the effects of aging, protection from calcium effects by certain binding reagents and the action of deoxycholate are comparable. It seems unlikely, however, that the inhibitory action of relaxing-factor preparations is apparent rather than real, owing to a resynthesis of ATP by oxidative-phosphorylating systems which may be present. Relaxing-factor action can be demonstrated in well-washed and aged preparations which would presumably be free from any endogenous oxidizable substrate. Further inhibition of adenosine triphosphatase occurs in the presence of 0·5 mM-2:4-dinitrophenol and in some cases can be demonstrated in the less-readily sedimented granular fractions which contain few mitochondria.

From the experimental data presented above it can be calculated that to bring about 50% inhibition of the adenosine-triphosphatase activity of 1 mole of myosin (mol.wt. 400 000) in the myofibrillar form 14 600 g. of total granular protein is required. As presumably only a fraction of this granular protein has factor activity it seems unlikely that it could depress the myofibrillar adenosine-triphosphatase activity by binding enough magnesium to alter the magnesium/ATP ratio to an inhibitory level, as was suggested earlier (Perry, 1956; Perry & Grey, 1956). A more plausible explanation is that the factor inhibits by binding the trace of metal which appears to be essential for the Mg^{2+}-ion-activated adenosine triphosphatase of myofibrils. Such a mechanism was suggested earlier to explain the inhibitory action of ethylenediaminetetra-acetate on the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase at concentrations which were only 1–2% of that of the added magnesium (Perry & Grey, 1956). Binding of small amounts of this essential metal by excess of ATP could also explain the substrate inhibition obtained with the Mg^{2+}-ion-activated myofibrillar adenosine triphosphatase. The fact that this substrate inhibition is relieved by traces of calcium which are in themselves insufficient to activate the enzyme (Perry & Grey, 1956) suggests that calcium is the cation required in trace amounts for the Mg^{2+}-ion-activated adenosine triphosphatase. Recently Weber (1959) has independently expressed such a view and provided some experimental evidence in support of it. Further evidence that there are special aspects of the Mg^{2+}-ion-activated hydrolysis of ATP by myofibrils which are of fundamental significance in the mechanism of the relaxing-factor action are the results with inosine triphosphate and uridine triphosphate. Inhibition of the hydrolysis of these nucleotides by excess of substrate is very much less marked than with ATP (Perry & Grey, 1956). Significantly in the present study it has been shown that the myofibrillar adenosine triphosphatase was inhibited by the factor preparations, whereas the inosine triphosphatase and uridine triphosphatase were not, under the conditions studied. Hasselbach (1956) showed that these two nucleotides did not induce relaxation in glycerated fibres.

Taking all these facts into consideration a possible mechanism for the inhibitory action of factor preparations towards adenosine triphosphatase is as follows. It is postulated that the factor has a strong affinity for the trace of metal (possibly calcium) which is essential for the Mg^{2+}-ion-activated adenosine triphosphatase of the myofibril. In resting muscle this is bound strongly and the myofibrillar adenosine triphosphatase is at a low activity. It appears that the requirement for this metal is a feature of substrates with the 6-amino group, for inhibition of inosine triphosphatase and uridine triphosphatase under otherwise identical conditions does not occur. On stimulation there is a momentary liberation of calcium which either provides directly the trace of metal required for the Mg^{2+}-ion-activated hydrolysis of

ATP or by saturating the binding centres of the factor displaces from it the trace of metal essential for that purpose. On return to the resting state all of the calcium or activating metal is once more rendered unavailable to the myofibrillar system.

SUMMARY

1. The bulk of the myofibrillar adenosine-triphosphatase-inhibitory activity was found to be associated with the heavier fractions sedimented from rabbit-muscle extracts. In such extracts highest specific adenosine-triphosphatase-inhibitory activity was associated with the granule fraction of highest oxidative activity.

2. Granules isolated from pigeon-breast muscle had a considerably lower specific adenosine-triphosphatase-inhibitory activity than those from rabbit muscle and the more active fractions were sedimented less readily.

3. Oxalate, pyrophosphate and citrate potentiated the adenosine-triphosphatase-inhibitory activity of granules; succinate, malonate, hypophosphite and sulphite had no such action.

4. The loss of inhibitory activity which occurred on aging at 0° and in the presence of low concentrations of calcium chloride could be reversed by oxalate.

5. Myofibrillar inosine-triphosphatase and uridine-triphosphatase activities were not inhibited by granular preparations which strongly inhibited adenosine-triphosphatase activity under otherwise similar conditions.

6. Synthetic and natural actomyosin adenosine-triphosphatase activities were inhibited by the granular preparations.

7. A hypothesis for the mechanism of inhibition is suggested on the basis of these findings and earlier studies on the myofibrillar adenosine triphosphatase.

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Tetrose Metabolism

1. THE PREPARATION AND DEGRADATION OF SPECIFICALLY LABELLED [^{14}C]TETROSES AND [^{14}C]TETRITOLS

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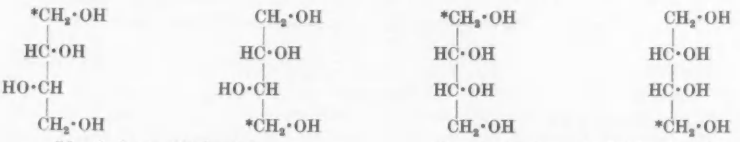
The fact that D-erythrose 4-phosphate arises in the hexose monophosphate pathway from the action of transketolase on fructose 6-phosphate, or of transaldolase on sedoheptulose 7-phosphate, has stimulated interest in tetrose metabolism, discussed in the subsequent paper (Batt, Dickens & Williamson, 1960*b*). The ease of synthesis of L-erythrulose from hydroxypyruvate in the presence of transketolase and a source of glycolaldehyde (Dickens & Williamson, 1956*a*; Dickens, 1958) provides a useful starting point for the production of a series of optically active ^{14}C-labelled tetroses and tetritols, with either specifically labelled hydroxy[^{14}C]pyruvate (Dickens & Williamson, 1958*a*) as the C_2-donor or hydroxy[^{12}C]pyruvate together with specifically labelled [^{14}C]glycolaldehyde as the acceptor aldehyde. Similarly, the aldolase-catalysed interaction of [^{14}C]formaldehyde with dihydroxyacetone phosphate (Charalampous, 1954; Peanasky & Lardy, 1958) yields a ketotetrose 4-phosphate; evidence in this paper indicates that this is the L-[4-^{14}C]erythrulose derivative, readily hydrolysable to give the free ketotetrose. L-Erythrulose is conveniently reduced by reduced diphosphopyridine nucleotide and the diphosphopyridine nucleotide-xylitol dehydrogenase of guinea-pig liver; the product with the mitochondrial enzyme is L-threitol (cf. Hollmann & Touster, 1957) and the activity of the similar enzyme purified from acetone-dried guinea-pig liver is approximately the same towards both D-xylulose and L-erythrulose (Hickman & Ashwell, 1959).

This paper describes the preparation, as a preliminary to the study of their metabolism, of various non-isotopic and specifically ^{14}C-labelled tetroses and tetritols. These include L-[1-^{14}C]- and L-[4-^{14}C]-erythrulose and D-[4-^{14}C]erythrose. In addition, preparations are given for L-threitol and *meso*erythritol (hereafter called erythritol) labelled with ^{14}C on one terminal carbon atom.

With such [^{14}C]polyols difficulties of nomenclature arise, which are discussed for the similarly labelled glycerols by Karnovsky, Hauser & Elwyn (1957). After considering possible alternatives, these authors prefer to refer to glycerol derived from D-[3-^{14}C]serine as L-[1-^{14}C]glycerol, and that from L-[3-^{14}C]serine as D-[1-^{14}C]glycerol, thus making the labelled carbon of the primary alcohol group of serine the C-1 of the polyol. It is this carbon atom of glycerol to which the phosphate group is attached in naturally occurring L-α-glycerophosphate, which is the exclusive product of the action of glycerokinase (Bublitz & Kennedy, 1954) and adenosine triphosphate upon biosynthetically prepared [1-^{14}C]glycerol. Glycerol, however, has the configuration Caa*bc, in which a* represents the isotopically labelled CH_2·OH group, and glycerol is therefore an 'isotopically asymmetric' compound, in the terminology of Karnovsky *et al.* (1957), although it is important to recognize that the asymmetry towards the enzyme is independent of the isotope labelling, being an example of the Ogston (1948) phenomenon, as in the analogous case of citric acid.

The general criteria for enzymic differentiation in such instances have been well reviewed by Schwartz & Carter (1954) and by Hirschmann (1956), who point out that this type of asymmetry is dependent on a plane bisecting the molecule through b and c, resulting in opposite ends which are related as mirror images. Hirschmann proposes an extension of this in the form of a new general rule which he also applies to the case Cabc-Cabc, as illustrated by the tartaric acids: 'If tartaric acid is considered as an intermediate in an enzymic process which results in differential labelling of the carboxyl groups, the symmetrical *meso* compound can qualify as a possible intermediate, but the dys-symmetric optically active forms cannot'. (Hirschmann, 1956, p. 167.)

This appears to apply to the tetritols also. Whereas each of the two optically active forms D- and L-threitol is expected, somewhat paradoxically on this view to behave identically at both ends, *meso*erythritol should be dissimilar at the two ends of the molecule. Since the configuration of the carbon atom adjacent to the final CH_2·OH group determines whether the compound is to be called



Identical L-[α-^{14}C]threitols (similarly for D-threitol)

Non-identical *meso*-[α-^{14}C]erythritols

D- or L-, this becomes obvious from consideration of the respective formulae when these are turned so as to make the labelled carbon atom C-1 (see above).

In this paper evidence supporting the identical enzymic behaviour towards the xylitol diphosphopyridine nucleotide dehydrogenase of L-[1-^{14}C]- and L-[4-^{14}C]-threitol is presented, in accordance with the above theoretical considerations. This is provided by the demonstration that when L-[4-^{14}C]-erythrulose is hydrogenated by the enzyme and reduced diphosphopyridine nucleotide, the product (which may be called either L-[1-^{14}C]threitol or L-[4-^{14}C]threitol) is capable of incorporating radioactivity into both C-1 and C-4 of an added pool of unlabelled L-erythrulose. This is evidence that both ends of the [^{14}C]threitol molecule are attacked by the enzyme, and since 80% of enzymic equilibration of C-1 and C-4 was reached there appears to be no enzymic distinction between the two terminal groups. It is suggested that this compound be designated L-[1-^{14}C(4-^{14}C)]threitol. Catalytic reduction of L-[1-^{14}C]erythrulose should similarly give L-[1-^{14}C]erythritol (i.e. D-[4-^{14}C]-erythritol), accompanied by L-[1-^{14}C]threitol; the separation of these reduction products by electrophoresis is described below.

In connexion with the tetrose syntheses, this paper also describes the preparation by means of D-amino acid oxidase from DL-[^{14}C]serine of hydroxy[^{14}C]pyruvate and L[^{14}C]serine. The latter is a useful source of [^{14}C]glycolaldehyde, which is produced by decarboxylation with ninhydrin.

A preliminary account of this work has been presented (Batt, Dickens & Williamson, 1960*a*).

ANALYTICAL METHODS

Formaldehyde. This was determined colorimetrically by the acetylacetone method of Nash (1953) and by the chromotropic acid method (Elwyn *et al.* 1957); also gravimetrically as the dimedone derivative.

L-Erythrulose. Enzymic oxidation of reduced diphosphopyridine nucleotide (DPNH) by diphosphopyridine nucleotide (DPN)-xylitol dehydrogenase (Hickman & Ashwell, 1959) was followed photometrically at 340 mμ. In pure solution L-erythrulose was determined by the measurement of hydrogen uptake, in the presence of platinic oxide catalyst, in Warburg manometers.

Tetritols. The formaldehyde liberated by periodate was determined by chromotropic acid.

Formic acid. This was also formed by periodate treatment, and was first reduced to formaldehyde (method of West & Rapoport, as modified by Faulkner, 1956) and the total resulting formaldehyde determined by comparison with a pure D-threitol standard. Alternatively, formate was oxidized to CO_2 (Pirie, 1946) and collected as $BaCO_3$.

Glucose. This was determined by the method of Nelson (1944).

Glycolaldehyde. The method of Dickens & Williamson (1958*b*) was used.

Glyoxylic acid. This compound, formed in periodate oxidation, was determined colorimetrically at 520 mμ on the solution of the 2:4-dinitrophenylhydrazone in N-NaOH (Friedemann, 1957), with a sodium glyoxylate standard. The method is not specific for any particular α-oxo acid.

Serine. The serine eluted from Amberlite IR-120 (H^+ form) resin was determined as formaldehyde liberated by periodate from the CH_2·OH group.

Glycollic acid. This was determined colorimetrically with 2:7-dihydroxynaphthalene.

Measurement of radioactivity. This was at infinite thickness with correction where necessary for self-absorption on 1 cm.^{2} disks in polythene planchets. Details were identical with those given by Dickens & Williamson (1959).

Total carbon of radioactive compounds. After oxidation with persulphate and silver nitrate, the CO_2 was converted into $BaCO_3$ for counting (Katz, Abraham & Baker, 1954).

High-voltage ionophoresis (Efron, 1959). Tetritol solutions were applied to the base line either as 5 μl. spots or for preparative purposes as a band 30 cm. wide on Whatman no. 1 papers, 42 cm. × 46 cm. Saturated aqueous boric acid solution, adjusted to pH 6·0 (Rammler & MacDonald, 1958), was the liquid phase. The current (120 mA at 4000 v) was applied for 2·5 hr. Tetritols were localized on marginal strips by the benzidine–periodate spray. We are much indebted to Dr Mary Efron for kindly allowing the use of her apparatus for the electrophoresis.

Paper chromatography. Descending liquid phase was used with Whatman no. 1 paper, the excess of liquid dripping from the free end. The usual experimental period was 16 hr. Solvents normally used were: A, butan-2-one–acetic acid–aq. 4% (w/v) boric acid (9:1:1); B, ethyl acetate–pyridine–water (12:5:4). Detection was either by dipping the dried paper in aniline–phosphoric acid–acetic acid solution (cf. Dickens & Williamson, 1956*b*), or, for polyols and sugars having adjacent hydroxyls, by the periodic acid–benzidine reagent (Gordon, Thornburg & Werum, 1956). The presence of radioactive spots was detected by a thin-window Panex monitor (model 50A), and for semi-quantitative purposes counts were made on squares 2 cm. × 2 cm. cut from the paper and measured in the standard lead castle fitted with a thin end-window counter and scaler (see Measurement of radioactivity, above).

Where necessary, phosphate esters were separated in methanol–aq. NH_3 soln. (sp.gr. 0·88)–water (6:1:3; cf. Charalampous & Mueller, 1953). Spots were developed by

Table 1. *Paper chromatography of tetroses and tetritols*

Solvent	Distance moved relative to glucose (R_G)*			
	L-Erythrulose	D-Erythrose	Erythritol	D-Threitol
A. Butan-2-one–acetic acid–aq. 4% boric acid (9:1:1)	8·9	10·1	5·9	6·0
B. Ethyl acetate–pyridine–water (12:5:4)	2·25	2·00	1·56	1·44

* These values are typical but markers should always be used for identification purposes.

molybdate spray and subsequent exposure to ultraviolet light.

Some typical data are included in Table 1.

MATERIALS

Radioactive substances. These were obtained from The Radiochemical Centre, Amersham, Bucks, with the following approximate specific activities: [^{14}C]paraformaldehyde, 1 mc/m-mole; sodium [3-^{14}C]pyruvate, 1 mc/m-mole; DL-[3-^{14}C]serine, 1 mc/m-mole; L-[3-^{14}C]serine, 3 mc/m-mole; D-[1-^{14}C]glucose and D-[6-^{14}C]glucose, 2 mc/m-mole.

Other substances. Recrystallized lithium hydroxypyruvate monohydrate was prepared as described by Dickens & Williamson (1958*a*). Lithium or sodium hydroxy[3-^{14}C]-pyruvate were similarly prepared from [3-^{14}C]pyruvate; the enzymic preparation is described below. L-Erythrulose was prepared as the crystalline *o*-nitrophenylhydrazone by the action on erythritol of *Acetobacter suboxydans*, N.C.I.B. 7069, kindly supplied by the Curator, National Collection of Industrial Bacteria, Torry Research Station, Aberdeen (cf. Whistler & Underkofler, 1938). L-Erythrulose solutions were prepared as required by refluxing the hydrazone with benzaldehyde and a little benzoic acid, followed by ether extraction of these substances from the cooled filtrate. The product was chromatographically pure and was used as a diluent for the ^{14}C-labelled erythrulose. The *cyclo*hexylammonium salt of fructose 1:6-diphosphate (McGilvery, 1953) was prepared from the barium salt.

L-Threitol was prepared enzymically as follows. L-Erythrulose (150 μmoles) was incubated at 37° in a total volume of 20 ml. containing DPN-xylitol dehydrogenase (1 ml.), DPN (10 mg.), liver alcohol dehydrogenase (0·2 ml., C. F. Boehringer und Söhne, Mannheim, Germany), aq. 0·02 N-NH_3 soln. (5 ml.) and ethanol (1 ml.). Disappearance of erythrulose was followed by photometric estimation of DPNH oxidation (see Analytical methods section) on samples incubated with DPN-xylitol dehydrogenase. No erythrulose remained after 90 min. and the whole solution was heated to 100° for 5 min., cooled, filtered, treated with Bio-Deminrolit G and evaporated to dryness *in vacuo*. The residue in 0·5 ml. of water was chromatographed with solvent B, the single band corresponding to the threitol marker was eluted and the eluate used directly as a solution of L-threitol.

D-Threitol was kindly provided by Professor J. H. Birkinshaw (Birkinshaw, Stickings & Tessier, 1948).

Commercial preparations purchased from C. F. Boehringer und Söhne were barium fructose 1:6-diphosphate, DPN and DPNH. Bio-Deminrolit G was supplied by The Permutit Co. Ltd. (see Dickens & Williamson, 1956*b*). Since Bio-Deminrolit G is no longer obtainable, its equivalent may be prepared by stirring 1 lb. quantities of Zeo-Karb 225 (H^+ form) with 2 N-HCl (2 l.) and De-Acidite G (Cl^- form) with aq. 2 N-NH_3 soln. (2 l.), washing the resins with distilled water until free of chloride, collecting the moist resins and thoroughly mixing. The resulting mixed-bed resin removes electrolytes with less than 5% loss of sugars. We are indebted to Dr T. R. E. Kressman of The Permutit Co. Ltd. for this suggestion.

Enzyme preparations. These were made as follows: transketolase (de la Haba, Leder & Racker, 1955) was prepared from The Distillers Co. Ltd. yeast, the stages of treatment with protamine and alumina being omitted; it was free from carboxylase activity and had 10 000 Racker units of activity/ml. of final solution or 1450 units/mg. of protein; DPN- and triphosphopyridine nucleotide (TPN)-xylitol dehydrogenase were from guinea-pig liver (Hickman & Ashwell, 1959); D-amino acid oxidase was prepared from pig kidney but otherwise as described by Negelein & Brömel (1939); it was purified to the end of Stage 2 of these authors and was devoid of catalase activity.

Dialysed muscle fractions. Rat skeletal muscle (90 g.) was chilled and mechanically macerated for 1 min. in ice-cold 0·01 M-$NaHCO_3$; the extract was filtered through gauze (700 ml.) and fractionated by additions of $(NH_4)_2SO_4$. Fraction 1 (0·45 saturation) and Fraction 2 (0·45–0·60 saturation) were dissolved in 2-amino-2-hydroxymethyl-propane-1:3-diol (tris) buffer (30 ml., 0·02 M, pH 7·6) and dialysed at 2° against distilled water before use. In some experiments similar preparations from guinea-pig muscle were used. Fraction 1 consists of crude phosphohexokinase. Fraction 2 is used here as crude aldolase, or, according to Charalampous (1954), crude phosphoketotetrose aldolase. The more recent work of Peanasky & Lardy (1958), already mentioned, makes it doubtful if the latter is a separate enzyme different from ordinary aldolase, but this question does not appear finally decided as yet. Fraction 2 also served as a phosphohexose-isomerase preparation.

A third muscle enzyme used was fructokinase, prepared as described by Staub & Vestling (1951). This enzyme, which is without action on glucose, did not phosphorylate L-erythrulose, although it was highly active towards fructose and adenosine triphosphate (ATP). Consequently it can be used to distinguish or separate these two ketoses.

Commercially obtained enzymes. These included liver alcohol dehydrogenase, α-glycerophosphate dehydrogenase and aldolase (C. F. Boehringer und Söhne), yeast hexokinase and intestinal alkaline phosphatase (Sigma Chemical Co., St Louis, Mo., U.S.A.).

PREPARATIONS

Hydroxy[3-^{14}C]*pyruvate and* L-[3-^{14}C]*serine from* DL-[3-^{14}C]*serine.* DL-[3-^{14}C]Serine (11·4 μmoles, 100 μc), together with DL-[^{12}C]serine (40 μmoles),

was incubated aerobically at 37° in Warburg vessels containing O_2 and pyrophosphate buffer (0·1 ml.; 0·1 M, pH 8·5) in the presence of D-amino acid oxidase (3 ml.) until uptake of O_2 became very slow (14·1 μmoles of O_2 equivalent to 28·2 μmoles of serine). Unlabelled L-serine (200 μmoles) was added, followed by 5 ml. of 0·5 N-H_2SO_4. After removal of the protein precipitate by centrifuging the supernatant contained approximately 10 μmoles of hydroxypyruvate by enzymic analysis (Dickens & Williamson, 1958*b*). Carrier lithium hydroxypyruvate (200 μmoles) was added and the solution was passed through an Amberlite IR-120 (H^+ form) column. The combined eluate and water washings (40 ml.) were extracted continuously with ethyl ether for 70 hr. After the extract had been shaken with a little water, the ether was removed under reduced pressure and the solution of the residue in water (10 ml.) was the preparation of hydroxy-[3-^{14}C]pyruvate used for the synthesis of L-[1-^{14}C]-erythrulose. By enzymic and colorimetric analysis (Dickens & Williamson, 1958*b*) the solution typically contained 125 μmoles (7·4 μc) of hydroxypyruvate. The loss is probably largely due to the alkaline incubation conditions (cf. Dickens & Williamson, 1958*a*).

The Amberlite column, after removal of the hydroxypyruvate as described above, was eluted with 50 ml. of aq. 2 N-NH_3 soln. The eluate was evaporated to dryness *in vacuo* and the residual L-[3-^{14}C]serine (179 μmoles, 40 μc) made up to 8 ml. with water

Several such batches of hydroxypyruvate (and L-serine) were freshly prepared as required.

Conversion of L-[3-^{14}C]*serine into* [2-^{14}C]*glycolaldehyde*. The solution of L-[3-^{14}C]serine (8 ml., 179 μmoles, 40 μc), prepared as described above, was mixed with 100 mg. of citrate [mixture of 19 g. of citric acid and 2 g. of trisodium citrate, well ground together (cf. Greenberg & Rothstein, 1957)] and 100 mg. of ninhydrin contained in a special 50 ml. conical absorption flask closed by a rubber seal (cf. Dickens & Williamson, 1959). The centre well contained 2 ml. of 2 N-NaOH (CO_2-free). The flask was evacuated and heated inside a protective container for 1·5 hr. at 100°, when the naphtharesorcinol test (Dickens & Williamson, 1958*b*) showed a 45% yield of glycolaldehyde. (The yield of CO_2 estimated as $BaCO_3$ was nearly quantitative and had only 1% of the total radioactivity.) The solution was deionized by Bio-Deminrolit G and concentrated to 5 ml.; 38·8% recovery of ^{14}C was found both by periodate and persulphate oxidations (see Analytical Methods section), indicating that all ^{14}C is in the CH_2·OH group.

L-[1-^{14}C]*Erythrulose and* L-[4-^{14}C]*erythrulose: synthesis by transketolase*. For the preparation of the 1-^{14}C-labelled compound, [3-^{14}C]hydroxypyruvate (96 μmoles, 5·9 μc; in 8 ml. of water) and [^{12}C]-glycolaldehyde (100 μmoles) were incubated at 37° in $N_2 + CO_2$ (95:5, v/v) with transketolase (1·75 ml.), phosphate buffer (2·5 ml.; 0·5 M, pH 6·2), thiamine pyrophosphate (2 mg.) and magnesium chloride (100 μmoles); total volume was 13·25 ml. A portion (2·65 ml.) was measured into a separate Warburg vessel and the CO_2 evolution followed manometrically. The whole was deionized with Bio-Deminrolit G, filtered, the filtrate was evaporated to dryness *in vacuo* and the residue of [1-^{14}C]erythrulose was redissolved in 5 ml. of water (4·5 μc, 66 μmoles of erythrulose).

Exactly the same procedure was used with [2-^{14}C]glycolaldehyde (87 μmoles, 15 μc; in 5 ml. of water), lithium hydroxy[^{12}C]pyruvate (100 μmoles), transketolase and other additions as described above. Evolution of CO_2 was 75 μmoles and after deionization the residue of L-[4-^{14}C]erythrulose was made up to 5 ml. of aqueous solution (12·3 μc in 75 μmoles).

Conversion of [^{14}C]*paraformaldehyde into* [^{14}C]-*formaldehyde*. [^{14}C]Paraformaldehyde (1·00 mg. = 28 μc) was weighed with addition of unlabelled paraformaldehyde (8·85 mg.) and 15 ml. of water into a flask with efficient reflux; the mixture was gently boiled under a fume hood for 2 hr. to effect complete solution and depolymerization. After distillation with small additions of water, 21 ml. of distillate contained 293 μmoles of formaldehyde (acetylacetone-analysis method) or 91% of the calculated yield.

Enzymic synthesis of [1-^{14}C]*fructose* 1:6-*diphosphate*. D-[1-^{14}C]Glucose (5·0 mg., 12·5 μc) was phosphorylated in a total volume of 6·8 ml. containing $NaHCO_3$ (0·6 ml., 0·15 M), $MgCl_2$ (0·2 ml., 0·1 M), sodium adenosine triphosphate (Sigma Chemical Co.; 0·6 ml., 0·1 M) and yeast hexokinase (Sigma Chemical Co.; 4 mg.). The reaction course was followed manometrically [at 37°, gas phase $N_2 + CO_2$ (95:5, v/v)]. When gas evolution was virtually complete, 1·0 ml. of N-HCl was added, the mixture was cooled to 0°, neutralized and the slight precipitate removed by centrifuging. After concentration *in vacuo* to 2·0 ml. the solution (now containing [1-^{14}C]glucose 6-phosphate and some [1-^{14}C]fructose 6-phosphate) was reincubated in a Warburg vessel under $N_2 + CO_2$ (95:5, v/v) at 37° with the following additions: $NaHCO_3$ (0·3 ml., 0·15 M), $MgCl_2$ (0·1 ml., 0·1 M), phosphohexose isomerase (0·05 ml. of dialysed muscle fraction 2, see Materials section), phosphohexokinase (0·10 ml. of dialysed muscle fraction I, see Materials section), sodium adenosine triphosphate (0·4 ml., 0·1 M). After 60 min. at 37°, 0·5 ml. of N-HCl and water (6 ml.) were added, and the mixture was neutralized and centrifuged from insoluble matter. The supernatant (10·0 ml.) was assayed for fructose 1:6-

diphosphate and triose phosphate by observation of the fall of the absorption at 340 mμ due to added DPNH (0·1 ml. of solution was taken for analysis: consecutive additions of α-glycerophosphate dehydrogenase, and then aldolase, in the spectrophotometer cuvettes were made as usual). The total volume (10·0 ml.) contained 13·7 μmoles of triose phosphate and a further 28·0 μmoles of triose phosphate were released on addition of aldolase, equivalent to 14·0 μmoles of fructose 1:6-diphosphate present. The glucose taken (5 mg.) was equivalent to 56 μmoles of triose phosphate; combined recovery was 75%.

Aldolase synthesis of [1-^{14}C]*erythrulose.* The above-mentioned solution (9 ml.) was used directly for the preparation of erythrulose 1-phosphate, since in the presence of aldolase and triose phosphate isomerase the entire material is available in the form of dihydroxyacetone phosphate for aldolase condensation with formaldehyde. For this purpose, unlabelled fructose 1:6-diphosphate (0·4 ml., 0·1 M), unlabelled formaldehyde (1·0 ml., 133 μmoles), water (6 ml.) and muscle fraction 2 (2·0 ml., see Materials section) were added and the solution was incubated at 37°. Samples (0·2 ml.) were removed at 0, 30 and 60 min. for estimation of formaldehyde and, after 60 min., the mixture was cooled to 0° and 4 ml. of perchloric acid was added, the precipitated protein was removed, the supernatant neutralized to pH 7·5 with 20% (w/v) KOH, and after it had been kept for several hours at 0° the $KClO_4$ was removed and the filtrate made to 30 ml. with water.

This solution containing erythrulose 1-phosphate was incubated with alkaline phosphatase (50 mg.) at 37° for 3 hr., then acidified with N-HCl (1 ml.) and the solution treated with Bio-Deminrolit G until neutral. The filtrate and water washings from the resin were concentrated *in vacuo* to 10 ml., containing [1-^{14}C]erythrulose (total radioactivity by persulphate oxidation 5·0 μC; radioactivity counted as the erythrulose phenylosazone 5·2 μC; mean 41% overall recovery of 1-^{14}C of the glucose taken).

Aldolase synthesis of L-[4-^{14}C]*erythrulose.* The preparation was similar to the one mentioned above, starting from unlabelled fructose 1:6-diphosphate (*cyclo*hexylammonium salt, 120 μmoles), [^{14}C]formaldehyde (126 μmoles, 11 μC), tris buffer (3 ml.; 0·2 M, pH 7·6), $MgCl_2$ (300 μmoles), muscle fraction 2 (5·0 ml.) in a total volume of 31·2 ml. The solution was incubated at 37°. In 3 hr. 70% of the formaldehyde had disappeared and the reaction rate had fallen sharply. Isolation of the product as described above gave 5 ml. of solution containing 80 μmoles (6·75 μC) of [4-^{14}C]erythrulose, i.e. 60% yield based on the amount of [^{14}C]formaldehyde taken. Chromatography in solvent A (see Analytical Methods section) and development by aniline phosphoric acid spray showed only one main spot corresponding to the erythrulose marker and a faint secondary spot corresponding to dihydroxyacetone.

Catalytic hydrogenation of the L-[^{14}C]*erythruloses.* Portions (0·8 ml.) of the above-mentioned erythrulose solutions were transferred to the two side bulbs of Warburg vessels, of which the main part contained platinic oxide monohydrate (5 mg., Johnson, Matthey and Co. Ltd.) in 2 ml. of water. After complete saturation of the catalyst with hydrogen by shaking at 37° in a stream of H_2 (30 min.) the taps were closed and the substrate was tipped in, the manometric readings being continued until hydrogenation was complete. The values observed with pure non-isotopic L-erythrulose agreed excellently with those calculated for 1 mole of H_2/mole of ketotetrose. The filtrate and washings were combined ('mixed tetritols').

Composition of the mixed tetritols. The reduction products from [4-^{14}C]erythrulose (and also from the pure unlabelled compound) were subjected to high-voltage ionophoresis in a water-cooled apparatus as detailed in the Analytical methods section. In the pH 6 borate medium clear separation occurred of two bands only, one moving 2 cm. towards the cathode (? electro-osmosis), the other 11 cm. towards the anode. From the behaviour of authentic non-isotopic markers, these were identified as erythritol (2 cm.) and threitol (11 cm.) respectively. The latter is evidently the L-isomer when derived from reduction of L-erythrulose; synthetic D-threitol was used for identification of this band. The two bands were separately eluted with water, the volumes of the eluates adjusted to 2·0 ml. and persulphate oxidations were made on 1·0 ml. portions, to which were added 100 μmoles of non-isotopic carrier erythritol. From the radioactivities of the resulting $BaCO_3$ it was calculated that the 'mixed tetritols' contained 72% of erythritol and 28% of L-threitol.

Enzymic preparation of L-[1-^{14}C]*threitol.* This substance was prepared from both L-[1-^{14}C]erythrulose and L-[4-^{14}C]erythrulose by the following method. The labelled erythruloses (200 μmoles, 5–10 ml., 5·0 μC) were incubated with enzyme and DPNH as described for the preparation of non-isotopic L-threitol (see Materials section). The isotopic yield averaged 50% in the final chromatographically pure products from either starting material.

Preparation of D-[4-^{14}C]*erythrose.* Perlin & Brice (1956) have described a convenient preparation of non-isotopic D-erythrose from D-glucose which we have applied to D-[6-^{14}C]glucose on a micro scale. To a solution of D-[6-^{14}C]glucose (200 μmoles, 10 μC) in 3 drops of water, 3 ml. of acetic acid and

200 mg. of lead tetra-acetate (L. Light and Co. Ltd.) were added with shaking at room temperature for 10 min. A solution of oxalic acid dihydrate (55 mg.) in a little acetic acid was added and, after 20 min., the precipitate of lead salt was removed and washed with 10 ml. of acetic acid. To the residue, after removal *in vacuo* of the solvent, 0·5N-H_2SO_4 (20 ml.) was added, followed by hydrolysis at 45° for 18 hr. The pH was then brought to 5·0 by addition of saturated $Ba(OH)_2$, the solution and washings of the $BaSO_4$ precipitate were combined and shaken with Bio-Deminrolit G (2 g.) for 30 min. The filtered solution was concentrated *in vacuo* to 2 ml. and then chromatographed on paper (solvent B) and chromatographed similarly again to separate from a faint adjacent band. Markers of authentic D-erythrose were prepared from crystalline 2:4-ethylidene D-erythrose made as described by Rappoport & Hassid (1951) for the preparation of the L-isomer. The eluted D-[4-^{14}C]-erythrose (70 μmoles) was made to 5 ml. with water. After addition of carrier erythrulose to 0·1 ml. samples, persulphate oxidation gave 3·5 μc of total $^{14}CO_2$, counted as $BaCO_3$, and periodate degradation of the phenylosazone (see erythrulose degradation, described below) showed that 0·8% of the total radioactivity was in C-1+C-2+C-3 and 99·2% of the ^{14}C was in C-4 (counted as formaldehyde dimedone).

DEGRADATIONS OF ^{14}C-LABELLED COMPOUNDS

Action of periodic acid on L-*erythrulose.* L-Erythrulose (120 μmoles) solution was measured into an absorption flask with 2N-NaOH as CO_2 absorbent in the centre well. After the addition of periodic acid (British Drug Houses Ltd., final concentration 0·3M) the stoppered flask was kept at room temperature for 50 min., when the $BaCO_3$ prepared from the alkali weighed 16·6 mg. (84 μmoles). Formic acid, formaldehyde and glycollic acid were also determined (see Analytical methods section). The reaction mixture was neutralized with 0·15M-$Ba(OH)_2$, the supernatant and washings of the precipitate were adjusted to pH 8·0 and the formaldehyde was distilled into 30 ml. of saturated dimedone solution: yield of crystalline dimedone derivative was 54·4 mg. The distillation residual solution was acidified with 1 ml. of 5N-H_2SO_4 and steam-distilled, and the distillate after neutralization was evaporated to small volume; the sodium formate was then oxidized with mercuric chloride–sodium acetate reagent to CO_2 (24·4 mg. as $BaCO_3$, 123 μmoles).

The residual solution from the steam-distillation was added to 15 ml. of 0·1N-ceric sulphate in an absorption flask having 2N-NaOH in the centre well. The evacuated stoppered flask was heated at 100° for 1 hr.; CO_2 was evolved (weighed as $BaCO_3$, 7·3 mg., 37 μmoles). No glyoxylic acid was detected in the periodate reaction mixture.

Two possible routes of oxidation (*A* and *B*) are consistent with these findings. A third route (*C*) can be excluded by the absence of glyoxylic acid formation:

Erythrulose carbon atom no.	C-1	C-2	C-3	C-4
A	CH_2·OH	CO_2H	H·CO_2H	H·CHO
B	H·CHO	CO_2	CHO	CH_2·OH
			↓	↓
			H·CO_2H	H·CHO
C	H·CHO	CO_2H	CHO	H·CHO

Reeves (1941) has shown that glycolaldehyde (the intermediate presumed to be formed from C-3 and C-4 in *B*) would be converted quantitatively into formic acid and formaldehyde. (Glycolaldehyde may similarly result from C-3 and C-4 in route *A* as an intermediate.) Pathway *B* is assumed proportional to the observed CO_2 evolution (70%) and pathway *A* to the glycollic acid formation (30%). On this basis the yields in μmoles/100 μmoles of erythrulose (recovery of total C of erythrulose 97·5%) were: CO_2, 70; glycollic acid, 30; H·CHO, 156; H·CO_2H, 104 (Calc.: CO_2, 70; glycollic acid, 30; H·CHO, 170; H·CO_2H, 100).

Although the above results are typical, individual variations in separate periodate oxidations occurred, suggesting that the extent of the two pathways is rather variable according to small experimental variations. Periodate oxidation of erythritol, on the other hand, gave consistent results; e.g. 100 μmoles of erythritol gave 176 μmoles of formaldehyde dimedone, and 182 μmoles of $BaCO_3$ from the mercuric acetate oxidation of formic acid. This corresponds respectively to 88% of formaldehyde recovered from C-1 and C-4 and 91% of formic acid from C-2 and C-3.

Degradation of erythrulose phenylosazone. In view of the variability of results with the free ketotetrose, the osazone was first prepared and then degraded by periodate oxidation. For example, a mixture of erythrulose (200 μmoles), sodium acetate trihydrate (1 g.) and phenylhydrazine hydrochloride (0·5 g.) in 3·5 ml. of water was heated at 100° for 30 min. The filtrate, together with 6·5 ml. of water washings, was cooled and the crystals of phenylosazone were collected (95%

yield). For recrystallization it was dissolved in a little hot ethanol, water was added to the filtered solution to slight turbidity and the solution was cooled.

Periodate oxidation of the erythrulose phenylosazone was as follows: the osazone (50 mg.) was dissolved in 24 ml. of warm 66% (v/v) ethanol. To the cooled solution, 0·2 ml. of N-$NaHCO_3$ and 0·8 ml of N-periodic acid were added. Rapid precipitation occurred of the bisphenylhydrazone of mesoxalaldehyde, identified by mixed m.p. with the same product derived from periodic acid oxidation of glucosazone. After 15 min. at room temperature, this was filtered on to weighed filter-paper disks (31·2 mg., 67% of calc.). To the filtrate and washings, saturated $Ba(OH)_2$ was added to pH 7–8 in order to remove excess of periodate. The supernatant and washings from this precipitate were combined and the formaldehyde was distilled into saturated dimedone solution (30 ml.). Yield of formaldehyde dimedone was 22·3 mg., or 46% of calc. for C-4 of the original erythrulose. This method is useful for determining the localization of radioactivity of C-1 + C-2 + C-3 (in the osazone) and C-4 (in the formaldehyde).

Localization of ^{14}C *in preparations of labelled erythrulose and tetritols.* Table 2 shows the results of degradation experiments carried out by the methods already described.

In the enzymic preparations an average of 92% of the total radioactivity appears in the expected carbon atoms of [1-^{14}C]- and [4-^{14}C]-erythrulose respectively. The extent of the randomization between these two positions, amounting variously from 2·6 to 12·8%, differs in the different preparations. Where transketolase is used, this enzyme is known to cleave L-erythrulose in the presence of an acceptor aldehyde, and a radioactive enzyme–glycolaldehyde complex has recently been shown to be formed when only [generally labelled (G)-^{14}C]-erythrulose or [G-^{14}C]fructose 6-phosphate is the substrate (Datta & Racker, 1959). Such a complex could effect a partial exchange reaction with unlabelled glycolaldehyde and thus introduce some labelling into the other C_2 moiety of erythrulose. In addition, some hydroxy[3-^{14}C]pyruvate will undergo spontaneous decarboxylation during incubation with transketolase and glycolaldehyde, yielding some [2-^{14}C]glycolaldehyde which can itself act as an acceptor aldehyde for hydroxy[3-^{14}C]pyruvate, yielding some [1:4-$^{14}C_2$]erythrulose. In the aldolase preparations also reported in Table 2, specificity of labelling was highest (97·4%) in the [4-^{14}C]erythrulose, as is to be expected since here H·^{14}CHO is the direct source of radioactivity and the dihydroxyacetone phosphate with which it combines is unlabelled. On the other hand the preparation of [1-^{14}C]erythrulose, by the similar reaction with unlabelled formaldehyde, involved a rather complicated series of steps starting with [1-^{14}C]glucose and resulting finally in aldolase cleavage of [1-^{14}C]fructose 1:6-diphosphate. Possibly at some stage of this preparation radioactive carbon was transferred from C-1 of the original hexose to formaldehyde, though such a reaction is not known to occur. Whether this is the true explanation or not, the labelling of C-4 of the erythrulose was appreciable (12·8%) when this method of synthesis was used.

In the degradation of the ketotetroses shown in Table 2, radioactivity at C-1, C-2 and C-3 is combined. However, after reduction to the tetritols the degradations, also in Table 2, show that C-1 or C-4 respectively has nearly all the total activity, only about 3–4% being present in C-2 and C-3 combined.

Table 2. *Degradations of erythrulose and tetritols*

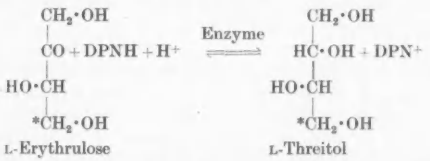
Substance degraded and method of preparation	Percentage of total ^{14}C	
	C-1 + C-2 + C-3*	C-4†
L-[1-^{14}C]Erythrulose		
Transketolase (prep. 1)	91·7	8·3
Transketolase (prep. 2)	90·0	10·0
Aldolase (prep. 3)	87·2	12·8
L-[4-^{14}C]Erythrulose		
Transketolase (prep. 4)	6·6	93·4
Transketolase (prep. 5)	11·0	89·0
Aldolase (prep. 6)	2·6	97·4
D-[4-^{14}C]Erythrose (chemical)	0·8	99·2
	C-1 + C-4‡	C-2 + C-3§
Tetritols from [1-^{14}C]erythrulose (prep. 1)‖	97·2	2·8
Tetritols from [4-^{14}C]erythrulose (prep. 4)‖	95·7	4·3

* As the osazone of mesoxalic semialdehyde.
† As formaldehyde from the osazone.
‡ As formaldehyde liberated by periodate.
§ As formic acid liberated by periodate.
‖ These tetritols were produced by catalytic hydrogenation (see text).

The labelling of substrates described in this paper is therefore sufficiently specific for their use in metabolic studies. These are described in the subsequent paper (Batt *et al.* 1960*b*).

TESTS WITH ENZYME SYSTEMS

Proof of isotopic symmetry of [1-^{14}C]- *and* [4-^{14}C]-L-*threitol.* The principle of this experiment is the study of distribution of ^{14}C between C-1 and C-4 of L-erythrulose when a pool of unlabelled L-erythrulose is incubated with DPN, DPN-xylitol dehydrogenase and L-[^{14}C]threitol:



L-Erythrulose L-Threitol

The terminal group of the threitol is that bearing ^{14}C. If in the reaction from right to left the enzyme does not distinguish between the two CH·OH groups of threitol, ^{14}C should be found after incubation in both C-1 and C-4 of the erythrulose pool. Preliminary studies showed that this equilibrium lies far to the right, as in the DPNH-catalysed D-xylulose–xylitol transformation studies by Hollmann & Touster (1957). For example, with 8 μmoles of L-threitol in the presence of 0·8 μmole of DPN$^+$ the DPNH formation was equivalent to 0·3% of the L-threitol added at pH 8·0. In a similar system with 10 μmoles of L-erythrulose added, only 0·15% of the L-threitol was oxidized as judged by DPNH formation.

To test ^{14}C incorporation the following system (total volume, 1·7 ml.) was maintained at room temperature for 2 hr.: L-[1-^{14}C]threitol, prepared enzymically from L-[4-^{14}C]erythrulose, 1·65 μmoles, 0·24 μc; unlabelled L-erythrulose, 5 μmoles; DPN-xylitol (D-xylulose) dehydrogenase 0·2 ml.; tris buffer (0·3 ml.; 0·5M, pH 8·0); $MgCl_2$ (0·3 ml., 0·1M) and DPN$^+$ (0·8 μmole). After 2 hr. reaction time, carrier L-erythrulose (150 μmoles), sodium acetate trihydrate (1 g.) and phenylhydrazine hydrochloride (0·5 g.) were added, with warming to 80° for 30 min. The osazone was collected after keeping at 0° overnight, washed, dried, counted, recrystallized and recounted. No fall of specific activity occurred on recrystallization and the total recovery of ^{14}C was 0·047 μc (20%). Oxidation with periodate of the recrystallized osazone (see Analytical methods section) showed that 42% of the total radioactivity appeared in C-1 + C-2 + C-3 and 58% in C-4 of the isolated tetrosazone. The original L-[4-^{14}C]erythrulose from which the L-[^{14}C]threitol was prepared had only 2·0% of its ^{14}C in C-1 + C-2 + C-3 and 98·0% of the total radioactivity in C-4. Hence, during the incubation with enzyme, 80% of complete exchange of isotopic labelling between C-1 and C-4 may be concluded. As discussed at the beginning of this paper, this behaviour is consistent with enzymic identity of the terminal halves of the L-threitol molecule, expected on theoretical grounds.

Substrate specificity of the triphosphopyridine nucleotide-xylitol (L-*xylulose*) *dehydrogenase.* This enzyme from mitochondrial fraction of guinea-pig liver was found by Hollmann & Touster (1957) to attack only xylitol and L-xylulose in the presence of TPN$^+$ or TPNH respectively, whereas the DPN-linked polyol dehydrogenase also described by these authors attacked a wide range of polyols (see also Hollmann, 1959). The fact that the DPN-xylitol (D-xylulose) enzyme also actively dehydrogenates L-threitol, which has close structural similarity to xylitol, led us to test the reactivity of the TPN-xylitol (L-xylulose) enzyme (prepared as described by Hickman & Ashwell, 1959) towards various substrates, including D-threitol (see Fig. 1). The results are shown in Table 3.

Hence D-threitol specifically requires TPN$^+$ and reacts quite vigorously with the TPN-xylitol (L-xylulose) dehydrogenase of guinea-pig liver. The expected dehydrogenation product is D-erythrulose, but this material was not available to us to test the reverse reaction. However, a specimen of

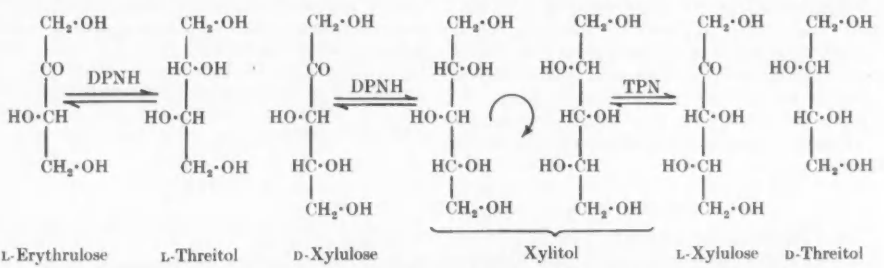


Fig. 1. Reactions of DPN-xylitol (D-xylulose) and TPN-xylitol (L-xylulose) dehydrogenases.

Table 3. *Changes in extinction with different substrates*

The enzyme (0·1 ml.) was tested with D-threitol, xylitol, erythritol or L-threitol (20 μmoles), TPN^+ or DPN^+ (0·8 μmole), in a total volume of 3·0 ml. containing tris buffer (30 μmoles), pH 8, and $MgCl_2$ (30 μmoles). Changes in extinction at 340 mμ after 20 min. at room temperature are recorded; the observations with TPN refer to the TPN-xylitol (L-xylulose) enzyme and in those with DPN the DPN-xylitol (D-xylulose) dehydrogenase was substituted under otherwise identical conditions. L-Threitol was enzymically prepared and the observation of reduction with both enzymes needs to be confirmed on chemically pure material.

Substrate	Increase in E	
	With DPN	With TPN
D-Threitol	0·009	0·120
Xylitol	0·380	0·254
Erythritol	0·004	0·003
L-Threitol	0·083	0·046

DL-erythrulose was prepared in poor yield from erythritol by the Fenton reaction (Neuberg, 1902), and purified by chromatography. The band running identically with pure L-erythrulose was eluted. The concentrated aqueous eluate reacted equally well with TPNH plus the TPN-xylitol (L-xylulose) dehydrogenase or with DPNH plus the DPN-xylitol (D-xylulose) dehydrogenase. It therefore contained, as expected, both isomers of erythrulose and the total content of DL-substance was determined in this way.

Since the TPN enzyme reduced this DL-erythrulose but not L-erythrulose, it may be concluded that D-erythrulose is a substrate for this enzyme, in addition to D-xylulose, for which according to Hollmann (1959) this enzyme is '*absolut specifisch*'.

Enzymic test of optical species of the erythrulose synthesized by aldolase. Erythrulose 1-phosphate was synthesized enzymically from dihydroxyacetone phosphate and formaldehyde, as described above: the free ketotetrose was liberated by phosphatase. Portions of the solution (1 μmole) added to the DPN-xylitol (D-xylulose) enzyme system (3 ml.) caused rapid oxidation of DPNH; authentic L-erythrulose behaved similarly. On the other hand, neither sample of ketotetrose caused any TPNH oxidation with the TPN-xylitol (L-xylulose)-dehydrogenase system. It may be concluded that the erythrulose synthesized by the aldolase reaction is present as L-erythrulose 1-phosphate. The optical species was not determined in the original preparation by this method (Charalampous & Mueller, 1953).

SUMMARY

1. The following preparations are described: (*a*) hydroxy[3-^{14}C]pyruvate and L-[3-^{14}C]serine from DL-[3-^{14}C]serine by D-amino acid oxidase; (*b*) [2-^{14}C]glycolaldehyde from [3-^{14}C]serine by decarboxylation with ninhydrin; (*c*) L-[1-^{14}C]-erythrulose and L-[4-^{14}C]erythrulose from the action of transketolase on the appropriately ^{14}C-labelled hydroxypyruvate and glycolaldehyde; (*d*) L-[4-^{14}C]erythrulose by the action of crude muscle aldolase on a mixture of H·^{14}CHO and dihydroxyacetone phosphate, followed by phosphatase hydrolysis of the erythrulose 1-phosphate so formed (it is shown enzymically that this compound is the L-isomer of erythrulose); (*e*) L-[1-^{14}C]-erythrulose similarly, by aldolase action on unlabelled formaldehyde and [1-^{14}C]dihydroxyacetone phosphate, prepared enzymically from D-[1-^{14}C]glucose.

2. L-[1-^{14}C]Threitol was prepared enzymically from either L-[1-^{14}C]erythrulose or L-[4-^{14}C]erythrulose, by means of reduced triphosphopyridine nucleotide and the 'diphosphopyridine nucleotide-xylitol (D-xylulose) dehydrogenase' of guinea-pig liver. The isotopic identity of L-[1-^{14}C]threitol and L-[4-^{14}C]threitol is experimentally proved, in confirmation of theoretical prediction. The correspondingly labelled erythritols are expected to be different compounds, but this remains to be investigated.

3. The preparation of D-[4-^{14}C]erythrose by periodate oxidation of D-[6-^{14}C]glucose is described.

4. By hydrogenation of the above-mentioned tetroses corresponding tetritols were prepared.

5. The position of isotopic labelling in the tetroses and tetritols has been determined by a study of the products of periodate oxidation of the free tetritols or of the phenylosazone of erythrulose (or erythrose).

6. The substrate specificity of the 'triphosphopyridine nucleotide-xylitol (L-xylulose) dehydrogenase' of guinea-pig liver has been shown to include D-threitol, as well as xylitol, for which substrate this enzyme was previously regarded as completely specific.

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Tetrose Metabolism

2. THE UTILIZATION OF TETROSES AND TETRITOLS BY RAT TISSUES

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Very little definite information is available about the metabolism of either tetroses or tetritols in the whole animal or in tissue preparations. This is surprising since there is now a considerable body of evidence that tetroses or their phosphates are capable of a variety of enzymic transformations. Thus the aldotetrose D-erythrose was first implicated in the pentose phosphate pathway of carbohydrate metabolism as a probable product, in the form of D-erythrose 4-phosphate, of the action of transaldolase on sedoheptulose 7-phosphate (Horecker & Smyrniotis, 1954; Horecker, Gibbs, Klenow & Smyrniotis, 1954; Horecker & Smyrniotis 1955; Horecker, Smyrniotis, Hiatt & Marks, 1955):

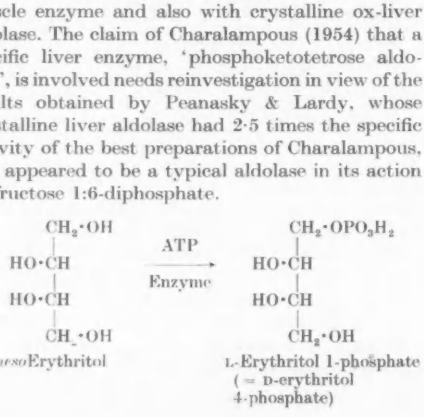
Sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate $\rightleftharpoons$ D-fructose 6-phosphate + D-erythrose 4-phosphate.

The reversibility of this reaction was considered probable; Racker, de la Haba & Leder (1954) were able to show that fructose 6-phosphate was itself split by transketolase yielding 'active glycolaldehyde' and again, presumably, D-erythrose 4-phosphate. The same product, together with dihydroxyacetone phosphate, was formed by the reversible cleavage by aldolase of sedoheptulose 1:7-diphosphate (Horecker *et al.* 1955; Smyrniotis & Horecker, 1956). Finally, Ballou, Fischer & MacDonald (1955) synthesized chemically D-erythrose 4-phosphate and showed that, with aldolase and dihydroxyacetone phosphate, sedoheptulose 1:7-diphosphate was formed. Kornberg & Racker (1955) showed that the synthetic tetrose ester behaved in the same

manner as the substance obtained as a barium salt (Srere, Kornberg & Racker, 1955) from the action of transketolase on fructose 6-phosphate with glyceraldehyde as 'acceptor aldehyde'.

L-Erythrulose is also a substrate for transketolase (Horecker & Smyrniotis, 1953; Horecker, Smyrniotis & Klenow, 1953); the latter paper, however, suggested that free L-erythrulose cannot be an intermediate in the synthesis by transketolase of heptulose phosphate from pentose phosphate, since added L-erythrulose did not become appreciably radioactive when [1-^{14}C]ribose 5-phosphate was the substrate, nor did it much dilute the isotope appearing in the sedoheptulose formed. The reverse reaction, the synthesis of L-erythrulose from transketolase acting on hydroxypyruvate with glycolaldehyde as acceptor, was shown by Dickens & Williamson (1956) [cf. Dickens (1958)] by isolation and determination of optical rotation of the recrystallized *o*-nitrophenylhydrazone of the product. L-Erythrulose is also reversibly reduced by the 'diphosphopyridine nucleotide-xylitol (D-xylulose) dehydrogenase' of guinea-pig liver in the presence of reduced diphosphopyridine nucleotide, as is fully described in the preceding paper (Batt, Dickens & Williamson, 1960*b*): the product is L-threitol.

Quite a different route for synthesis of ketotetrose 1-phosphate in liver was discovered by Charalampous & Mueller (1953) and Charalampous (1954), who found that an aldolase-like enzyme from liver causes condensation of dihydroxyacetone phosphate with formaldehyde, yielding an erythrulose 1-phosphate; we have presented evidence (Batt *et al.* 1960*b*) that this is also the L-erythrulose derivative. It was shown much earlier (Meyerhof, Lohmann & Schuster, 1936; cf. Lohmann, 1941) that a ketotetrose 1-phosphate resulted from this reaction when catalysed by crude muscle aldolase, and recently (Peanasky & Lardy, 1958) this has been confirmed with the once-crystallized rabbit-muscle enzyme and also with crystalline ox-liver aldolase. The claim of Charalampous (1954) that a specific liver enzyme, 'phosphoketotetrose aldolase', is involved needs reinvestigation in view of the results obtained by Peanasky & Lardy, whose crystalline liver aldolase had 2·5 times the specific activity of the best preparations of Charalampous, and appeared to be a typical aldolase in its action on fructose 1:6-diphosphate.

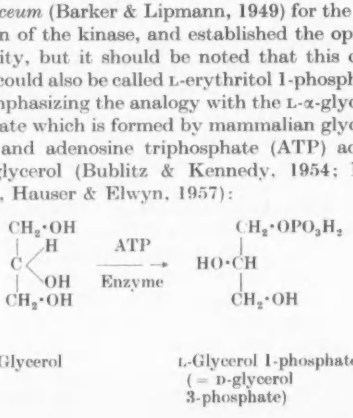


*meso*Erythritol — L-Erythritol 1-phosphate (= D-erythritol 4-phosphate)

The interesting possibility that a diphosphate of a tetrose might be a regulator of the activity of glyceraldehyde 3-phosphate dehydrogenase, which it powerfully and specifically inhibits, has been raised by recent work by Racker, Klybas & Schramm (1959): the substance appears to be D-threose 2:4-diphosphate (Fluharty & Ballou, 1959). D-Erythrose 4-phosphate strongly inhibits phosphoglucose isomerase (Grozi, De Flora & Pontremoti, 1960).

In contrast with these enzymic studies showing varied activities of tetroses and tetritols, metabolic studies on these substances are largely lacking. Erythritol, as the most available substance in this series, has received some attention (reviewed by Carr & Krantz, 1945). Beck, Carr & Krantz (1938) showed that erythritol and its anhydride, erythritan, were absorbed readily from the gastrointestinal tract of rats, were excreted in the urine in large amounts and were not precursors of liver glycogen in the fasting hen or rat. Meyer, McTiernan & Slater (1934) observed negligible anaerobic acid formation from erythritol by slices of mouse sarcoma 180. The urinary excretion in a normal man of erythritol and its oxidation by crude liver extracts has been briefly reported (Touster, Hecht & Todd, 1959). Metabolic studies on D- and L-threitol, the two optical isomers of erythritol, have not been made, according to Carr & Krantz (1945, p. 180) who state that in their opinion 'these substances will be found refractory to metabolism by the animal body'. This view, which appears to be generally held, is shown in the present work not to be entirely correct.

No studies on the phosphorylation in the animal body of these compounds have been found, but both the chemical and enzymic syntheses of D-erythritol 4-phosphate have been described by MacDonald, Fischer & Ballou (1956) and Shetter (1956) respectively. The latter used extracts of dried erythritol-adapted cells of *Propionibacterium pentosaceum* (Barker & Lipmann, 1949) for the preparation of the kinase, and established the optical specificity, but it should be noted that this compound could also be called L-erythritol 1-phosphate, thus emphasizing the analogy with the L-α-glycerophosphate which is formed by mammalian glycerokinase and adenosine triphosphate (ATP) acting upon glycerol (Bublitz & Kennedy, 1954; Karnovsky, Hauser & Elwyn, 1957):



Glycerol — L-Glycerol 1-phosphate (= D-glycerol 3-phosphate)

The 'isotopic asymmetry' of the two [α-^{14}C]erythritols, and the identity of each of the two D- and L-[α-^{14}C]threitols has already been discussed (Batt *et al.* 1960*b*). In the present work only the metabolism of the [1-^{14}C]erythritol produced by catalytic hydrogenation of L-[4-^{14}C]erythrulose (D-[1-^{14}C]erythritol, in the above-mentioned nomenclature) has been studied; it appeared not to be metabolized to radioactive carbon dioxide or glucose by rat-liver slices.

By the methods of preparation, identification and degradation described by Batt *et al.* (1960*b*) we have also investigated the metabolism in the whole rat and in rat-liver slices of the following specifically labelled tetroses and tetritols: in the whole rat, L-[1-^{14}C]- and L-[4-^{14}C]-erythrulose, and in rat-liver slices both of these substrates and also D-[4-^{14}C]erythrose, as well as a number of specifically ^{14}C-labelled tetritols. The percentage of radioactivity appearing in respiratory carbon dioxide, in glucose (or liver glycogen, in the intact rat), and in some cases in the urinary excretion of isotope, have been determined. In addition, the glycogen formed from L-[1-^{14}C]- and L-[4-^{14}C]-erythrulose has been hydrolysed and the localization of ^{14}C in the individual glucose carbon atoms has been determined. Similar degradations have been made on the glucose formed on incubation of these substances, and also of D-[^{14}C]erythrose, with liver slices. A brief account of this work has been presented (Batt, Dickens & Williamson, 1960*a*).

EXPERIMENTAL

Materials and analytical methods. These were as already described (Batt *et al.* 1960*b*). In addition, methods of animal experiments and general outlines of procedure, including measurement of radioactivity, were the same as those detailed by Dickens & Williamson (1959) for studies on hydroxypyruvate metabolism in the whole rat and in tissue slices. The following points are additional to those given in that paper.

Degradation of glucose for determination of location of ^{14}C. In the previous work (Dickens & Williamson, 1959), two methods were used for this purpose: fermentation by *Leuconostoc mesenteroides*, which yields isotope values for each of the six glucose carbon atoms, and yeast fermentation, which is quantitative only for determination of C-3 and C-4 of glucose.

In the present work difficulties were encountered in the use of *L. mesenteroides*. The amounts of radioactivity in the glucose were not sufficient to permit its recrystallization, and it is thought that purification by removal of ionized substances by Bio-Deminrolit G followed by paper chromatography was insufficient to remove some persistent impurity. Consequently, although satisfactory recoveries of radioactivity continued to be obtained with pure test samples of specifically labelled glucose, in experiments where glucose formation from tetroses (particularly L-erythrulose) was being studied in tissue slices, the glucose fraction analysed for radioactivity by *L. mesenteroides* showed rather inconsistent results. In particular, the values obtained for labelling at C-4 of glucose were occasionally suspiciously high. One such example is cited in the preliminary publication, in the experiment with L-[4-^{14}C]-erythrulose (Batt *et al.* 1960*a*).

In order to avoid this source of uncertainty, two alternative methods of degradation of glucose have been successfully applied. The former (Topper & Hastings, 1949) was purely chemical, beginning with the preparation of phenylglucosazone from the sample, a step which, together with the recrystallization of this compound, effects sufficient purification for reliable results to be obtained. Periodate oxidation of the recrystallized glucosazone yields C-1, C-2 and C-3 of glucose in the form of the phenylosazone of mesoxalic semialdehyde (Topper & Hastings, 1949), the radioactivity of which is measured and compared with the total activity of the recrystallized glucosazone. Also formed by the periodate oxidation are two molecules of formic acid from C-4 and C-5, and one molecule of formaldehyde from C-6. The former is converted into $BaCO_3$ and the latter into the dimedone compound for counts of specific activity. Details of procedure are given by Batt *et al.* (1960*b*).

For control of this procedure, D-[1-^{14}C]- and D-[6-^{14}C]-glucose (obtained from The Radiochemical Centre, Amersham, Bucks.) were appropriately diluted with unlabelled glucose. The subsequent analyses are summarized in Table 1.

Degradation methods and results for the other substrates used have already been described in detail (Batt *et al.* 1960*b*).

The above-mentioned methods of degradation were those mainly used, but a few experiments made use of *Lactobacillus casei* fermentation (Bernstein & Wood, 1957). Here the glucose molecule is symmetrically cleft into two molecules of lactate, which are then chemically degraded. As a result the methyl groups appear as the sum of C-1 and C-6, the secondary alcohol groups as the sum of C-2 and C-5 and the carboxyl groups as the sum of C-3 and C-4, all being counted as $BaCO_3$. Here also satisfactory control analyses on pure samples of labelled glucose were obtained. Because of the complications caused by tetrose metabolites in purification of glucose for the other methods, the precipitation as the osazone and subsequent degradation by periodate oxidation were generally preferred.

Procedure with intact rats. Male albino rats of the Wistar strain (wt. approx. 200 g.) were fasted for 18 hr., injected

Table 1. *Degradation of specifically labelled* [^{14}C]*glucose by periodate oxidation of glucosazone*

Substance	C-1, C-2, C-3	C-4, C-5	C-6	Overall recovery (%)
D-[1-^{14}C]Glucose	97·8	0·4	1·8	95·0
D-[6-^{14}C]Glucose	3·2	5·0	91·8	88·0

intraperitoneally with a trace dose (e.g. 10 μmoles, 1·5 μc) of the labelled tetrose or tetritol, followed by administration by stomach tube of 1 g. of glucose in 3 ml. of water. The rat was placed in a metabolism chamber consisting of a large desiccator with a top tubulure through which a stream of air was continuously drawn, after being freed from CO_2 by passage through large sintered-glass 'bubblers' containing 2N-NaOH solution. The rate of passage of gas was followed on a flow meter, and the effluent gases were passed through two more sintered-glass scrubbers to collect the respiratory CO_2 in NaOH solution free from carbonate. This apparatus was developed in conjunction with Dr G. E. Glock and Dr P. McLean, and it also permitted the separate collection of the rat's urine excreted in the 3 hr. period. The CO_2 was usually collected after each hour for a total period of 3 hr., when the animal was removed and killed, the liver being at once dissected for analysis. For this purpose the weighed liver tissue was first macerated for 1 min. with 15 vol. of 7% trichloroacetic acid and 2 g. of Celite 535, and stirred at 0° for 15 min. The glycogen precipitated from the filtrate by the addition of 1·1 vol. of ethanol was collected after 16 hr. at 0°, washed, dried, weighed and plated on planchets for determination of radioactivity. The glycogen was then hydrolysed by 2N-H_2SO_4 (3 hr. at 100°), the solution deionized with Bio-Deminrolit G and the resulting glucose solution treated with phenylhydrazine for the preparation and recrystallization of the osazone, as described in the references quoted above. In some experiments the glucose solution was first concentrated, streaked upon filter paper for chromatographic purification and eluted with water from the band cut from the paper (see Batt *et al.* 1960*b*).

The urine collected from the rat-metabolism chambers was measured and the total ^{14}C determined on a small portion by oxidation with the persulphate–$AgNO_3$ reagent to $^{14}CO_2$, followed by its conversion into $Ba^{14}CO_3$ for counting (Katz, Abraham & Baker, 1954). After deionization with Bio-Deminrolit G, the solution and washings were concentrated *in vacuo* and chromatographed as a band on paper with ethyl acetate–pyridine–water (12:5:4) for 16 hr. Suitable markers were run simultaneously, and the band corresponding to an erythritol marker was eluted, the eluate made to 10 ml. with water and the total $^{14}CO_2$ liberated from a 5 ml. portion was determined as before by persulphate oxidation. This is a measure of the excreted tetritols. A large part (average 75%) of the excreted ^{14}C-labelled compounds was removed by the treatment with the mixed-bed resin: the nature of this fraction remains to be investigated.

For the identification of the urinary tetritol, apart from the chromatography which identifies it as either erythritol or threitol (not separated in the solvents used; Batt *et al.* 1960*b*), electrophoresis on paper in borate at pH 6 (cf. Batt *et al.* 1960*b*) was also employed. The results (see below) were consistent with the expected nature of this product as threitol, presumably L-threitol produced by diphosphopyridine nucleotide (DPN)-xylitol dehydrogenase (Batt *et al.* 1960*b*).

Experiments with liver slices. Freshly excised liver from fed Wistar rats was sliced and divided into 1 g. batches of slices, which were incubated at 37° with shaking in Krebs–Ringer solution in $O_2 + CO_2$ (95:5) exactly as described by Dickens & Williamson (1959), except for the substrate, which consisted of the labelled tetrose or tetritol as shown in the tables. The subsequent liberation and collection as $BaCO_3$ of the CO_2, deproteinization and purification of the glucose contained in the solution were as described by Dickens & Williamson (1959). In addition to the treatment with Bio-Deminrolit G, the filtrate and water washing from the resin were concentrated *in vacuo*, usually to 0·5 or 1·0 ml., and the concentrate was chromatographed on paper [ethyl acetate–pyridine–water (12:5:4)] with suitable markers. The glucose area was cut and eluted with water, the eluate made to 10 ml., and unlabelled carrier glucose (50 mg.) was added after estimation of the glucose content by the method of Nelson (1944). The bands corresponding to the marker erythritol (and in some experiments also marker erythrulose) were also eluted similarly. These fractions are referred to as 'tetritol' and 'ketotetrose' in the Tables. The total ^{14}C in each of these fractions was determined after addition of carrier erythritol or erythrulose, by oxidation to CO_2 of measured portions by means of the persulphate–$AgNO_3$ reagent (Katz *et al.* 1954), conversion into $BaCO_3$ and plating on planchets for determination of radioactivity.

After the total radioactivity in a portion of the purified glucose fraction had been similarly determined, the osazone was prepared from this fraction (Batt *et al.* 1960*b*) and its radioactivity was determined before and after crystallization. The osazone was then degraded by periodate oxidation as already described.

RESULTS

Metabolism of L-[1-^{14}C]- *and* L-[4-^{14}C]-*erythrulose in the intact rat*

The data for rats injected with L-[1-^{14}C]erythrulose (dose 9–10 μmoles; 1·38 μc) and L-[4-^{14}C]-erythrulose (8–9 μmoles; 1·75 μc) are collected in Table 2. Whereas an average of 25·4% of the 1-^{14}C appeared in the respiratory CO_2 during the 3 hr. of the experiments, only 15·5% of the 4-^{14}C was converted into CO_2 in closely similar conditions. Incorporation of radioactivity into liver glycogen was, however, slightly higher (4·8% of the dose) with the 4-^{14}C compound than with the 1-^{14}C one (3·8%). Amounts between 11 and 26% of the dose appeared in the urine with the two substances, these figures being more variable than the rather constant values for glycogen and CO_2 incorporation. With both the [1-^{14}C]- and [4-^{14}C]-erythrulose, only about one-fifth of the total urinary excretion is due to tetritols. The fact that there was very little radioactivity at any point on the paper other than that at which the tetritols were found, suggests strongly that unknown ionized components of the urine, removed by the treatment with ion-exchange resin, comprise the other main urinary metabolites.

The total percentage of the dose accounted for is, however, considerable (45% for L-[1-^{14}C]erythrulose, 40% for L-[4-^{14}C]erythrulose; see Table 2).

Table 3 includes the results obtained on the localization of labelling in glucose carbon atoms

of glycogen by means of periodate degradation of the purified glucosazones. An average of 83% of the total radioactivity was recovered in the carbon atoms evaluated. Results of separate experiments with different rats were highly consistent and showed a significantly different pattern of labelling of glucose with the two differently labelled erythruloses. That with the [4-^{14}C]-erythrulose was more symmetrical, activity in C-1, C-2 and C-3 of glucose being about equal to that in C-6. Most of the activity in the first three carbon atoms is thought, by analogy with results reported later with liver slices, to be contained in C-1, which would thus become approximately equally labelled to C-6.

With the 1-^{14}C-labelled substrate, about twice as much radioactivity appears in C-1, C-2 and C-3 (again probably mainly in C-1) as is present in C-6 of the glucose isolated from the liver glycogen.

With either isotope labelled erythrulose, very little activity (total 5–13%) appears in C-4 and C-5 of the glucose formed.

The respiratory CO_2 (3 hr. totals only are shown in Table 2) was sampled at 1 hr. intervals; on the average, with [1-^{14}C]erythrulose, 45% of the $^{14}CO_2$ expired in the 3 hr. period appeared in the first hour, and 35 and 20% in the second and third hours, showing that the oxidative metabolism continued throughout this period at a steadily diminishing rate. With [4-^{14}C]erythrulose a different picture resulted, 25% being expired in the first hour and a peak of 45% being reached in the second hour, falling to 30% in the third hour of the experiment. The total elimination of $^{14}CO_2$ was also much higher with the 1-^{14}C-labelled compound, being 25·4% of the total dose of radioactive carbon, as compared with 15·5% for the 4-^{14}C-labelled compound.

Table 2. *Utilization of specifically labelled* L-*erythrulose in the intact rat*

Rat no.	Wt. of liver glycogen (mg.)	Percentage of dose of ^{14}C: In $^{14}CO_2$	In glycogen	In urine	In urinary tetritol	Total accounted for (%)
		L-[1-^{14}C]Erythrulose (dose 10 μmoles, 1·38 μc)				
1	124	25·0	3·0	18·8	4·0	46·8
2	87	23·4	2·4	17·4	—	43·2
3	131	23·8	4·6	14·5	7·9	42·9
4	190	29·4	5·1	10·9	2·6	45·4
Average	133	25·4	3·8	15·4	4·8	44·6
		L-[4-^{14}C]Erythrulose (dose 8–9 μmoles, 1·75 μc)				
5	88	18·4	4·2	25·7	—	48·6
6	112	13·2	5·4	24·0	3·1	42·6
7	100	15·6	4·2	16·6	3·7	36·8
8	63	14·6	5·4	13·1	3·5	33·0
Average	91	15·5	4·8	19·9	3·4	40·3

Table 3. *Distribution of* ^{14}C *in liver glycogen from rats injected with labelled* L-*erythruloses*

Rat numbers are the same as those of Table 2. Liver glycogen was isolated, hydrolysed, and glucosazone degraded (see text).

Rat no.	Percentage of total activity in glucose C atoms: C-1 + C-2 + C-3	C-4 + C-5	C-6	Total ^{14}C recovery on degradation (%)
	L-[1-^{14}C]Erythrulose			
1	61	13	26	83
2	64	13	23	83
3	59	11	30	94
4	64	6	29	80
Average	62	11	27	85
	L-[4-^{14}C]Erythrulose			
5	49	10	41	84
6	53	5	42	84
7	43	10	47	83
Average	48	8	45	84

Nature of urinary tetritols

In order to obtain sufficient material to ascertain the nature of urinary tetritols, half-portions of each of the chromatographically separated tetritol fractions from the urine of four rats which had received injections of L-[1-^{14}C]erythrulose (see Table 1) were pooled, evaporated to 0·5 ml. *in vacuo* and streaked on Whatman no. 1 paper (24 cm. × 20 cm.). Markers of erythritol and threitol were applied at each side, and the paper was subjected to electrophoresis in saturated aqueous boric acid adjusted to pH 6·0 with 10N-NaOH; 400v, 10 mA; 17 hr. at 4°. Erythritol moved 8 mm. towards the cathode, threitol and marginal spots of the urinary tetritol moved 32 mm. towards the anode (periodate–benzidine dip was used on marginal strips for localization). The initial total radioactivity of the pooled fractions (determined before concentration on one-tenth of a portion) was 0·12 μc. The total radioactivity eluted from the cut-out band corresponding with the threitol marker gave a recovery of 42% as [^{14}C]threitol. No other spots were detectable on the paper after the electrophoresis and it is concluded that the urinary tetritol was exclusively threitol.

Metabolism of tetroses and tetritols in liver slices

Table 4 shows the percentage of tracer doses of radioactivity converted into $^{14}CO_2$, [^{14}C]glucose and [^{14}C]tetritol on incubation with rat-liver slices (see Experimental section) of the following substances: L-[1-^{14}C]erythrulose (10 μmoles, 0·45 μc/vessel);

Table 4. *Metabolism of tetroses and tetritols in rat-liver slices*

For details of substrates see text. Period: 90 min. at 37°; $O_2 + CO_2$ (95:5). Respiratory CO_2, glucose, threitol and, in some experiments, erythrulose were isolated from incubation mixture after shaking 1 g. (wet wt.) of slices with substrate as shown.

	Percentage of added ^{14}C found in			
Rat no.	Resp. CO_2	Glucose	Tetritol	Erythrulose
Substrate: L-[1-^{14}C]Erythrulose (amount per vessel: 10 μmoles, 0·45 μc)				
8	13·9	4·8	—	—
9	9·6	6·2	19	18
10	14·9	4·5	13	—
11	12·0	4·3	—	6
12	8·9	3·7	8	—
13	12·9	2·3	6	—
Average	12·0	4·3	11·5	12
Substrate: L-[4-^{14}C]Erythrulose (amount per vessel: 10 μmoles, 1·2 μc)				
14	4·9	1·4	—	—
9	6·9	2·5	22	18
10	7·1	3·8	12	16
11	4·9	2·8	—	9
12	3·7	1·9	10	—
13	4·1	2·4	5	—
Average	5·3	2·4	12	14
Substrate: D-[4-^{14}C]Erythrose (amount per vessel: 10 μmoles, 0·48 μc)				
15	1·3	3·6	—	—
15	0·7	1·7	—	—
Substrate: Tetritols from reduction of labelled L-erythruloses				
14[a]	1·0	0·50	—	—
14[b]	0·9	0·75	—	—
17[c]	1·2	0·50	—	—
18[d]	0·0	0·04	—	—
19[e]	1·0	0·10	—	—
19[f]	1·4	0·13	—	—
20[e]	2·0	0·35	—	—
20[f]	1·1	0·30	—	—

[a, b] Substrate, 10 μmoles (0·48 μc) of catalytic hydrogenation product from L-[1-^{14}C]erythrulose (example of duplicate batches of slices).

[c] 6 μmoles (0·50 μc) of tetritols as described above from hydrogenation of L-[4-^{14}C]erythrulose.

[d] 6 μmoles (0·69 μc) of electrophoretically separated erythritol fraction from catalytic hydrogenation of L-[4-^{14}C]-erythrulose.

[e] Product of enzymic reduction of L-[1-^{14}C]erythrulose; 5·1 μmoles (0·68 μc) = L-[1-^{14}C]threitol.

[f] Product of enzymic reduction of L-[4-^{14}C]erythrulose; 5·0 μmoles (0·71 μc) = L-[1-^{14}C]threitol.

L-[4-^{14}C]erythrulose (10 μmoles, 1·2 μc); the mixed tetritols formed by catalytic hydrogenation of each of the above ketotetroses (see Batt *et al.* 1960*b*) containing 0·48–0·69 μc in 6–10 μmoles of total tetritol; and electrophoretically purified erythritol isolated from the catalytic hydrogenation, as described above, of L-[4-^{14}C]erythrulose. Finally, L-[1-^{14}C]threitol, produced by reduction of labelled L-erythrulose with reduced diphosphopyridine nucleotide (DPNH) and the DPN-xylitol (D-xylulose) dehydrogenase, was tested as substrate; it was shown in the preceding paper that this product is formed either from L-[1-^{14}C]erythrulose or L-[4-^{14}C]erythrulose, both of which give the same ^{14}C-labelled compound on enzymic reduction by this enzyme (Batt *et al.* 1960*b*).

Preliminary experiments on slices of liver tissue of two rats, incubated with D-[4-^{14}C]erythrose, are also included in Table 4; this substance was prepared chemically from D-[6-^{14}C]glucose (Batt *et al.* 1960*b*) and used in amounts of 10 μmoles (0·48 μc)/vessel.

Of the two labelled ketotetroses included in Table 4, the 1-^{14}C-labelled compound is much the richer source of respiratory $^{14}CO_2$ (average 12·3% compared with 5·2% for the 4-^{14}C-labelled compound), though it contributes about the same radioactivity (av. 12%) to the tetritol (presumably L-threitol), which accumulates in the incubation medium: the amount of the latter is, however, very variable in individual experiments. The incorporation into glucose of ^{14}C also varies considerably from rat to rat, but tends to be higher, sometimes twice as high, with the [1-^{14}C]ketotetrose. The extent of incorporation (1·3–6% of the dose) was in a similar range to that observed for D- and L-serine (Dickens & Williamson, 1959) and for a number of related compounds cited in that paper.

Table 4 shows also the behaviour with liver slices of D-[4-^{14}C]erythrose. The amount of radioactivity appearing in the respiratory CO_2 is much less than with either of the labelled ketotetroses (average 1·1%), whereas the incorporation into glucose is in the two experiments (average 3%) about the same. It would be desirable to extend these experiments with 1-^{14}C-labelled erythrose but this compound was not available.

By comparison with the above keto- and aldotetroses, the tetritols studied appear to be poorly metabolized.

The catalytic hydrogenation (Batt *et al.* 1960*b*) of L-erythrulose produces a mixture consisting of about 75% of erythritol and 25% of L-threitol. The erythritol fraction isolated from the mixture by high-voltage electrophoresis (Table 4, rat 18[d]) did not appear to be utilized appreciably, since ^{14}C from the 4-^{14}C-labelled compound contributed no detectable radioactivity to the respiratory CO_2 and a very low amount (0·04% of the total activity) to the glucose isolated after incubation.

The catalytic reduction products from L-[1-^{14}C]-erythrulose (rat 14, duplicates 14[a] and 14[b]) were, however, incorporated appreciably (1% as $^{14}CO_2$, 0·6% as [^{14}C]glucose). Closely similar figures were obtained with the mixed tetritols from L-[4-^{14}C]-erythrulose (rat 17[c]; in this and the following examples only means of duplicate batches of slices, which agreed well, are reported). This similarity is understandable since both substances give the same threitol (L-[1-^{14}C]- or L-[4-^{14}C]-threitol, which are identical: Batt *et al.* 1960*b*). Presumably only the threitol component is metabolized, though poorly. This view was confirmed by studies on the product of enzymic reduction of the two labelled L-erythruloses, prepared by means of DPNH and the DPN-xylitol (L-xylulose) dehydrogenase (rats 19 and 20[e] and 20[f]). These products have been shown by specific degradation methods (Batt *et al.* 1960*b*) to have 90% or more of the isotopic labelling in the named carbon atoms.

Isotopic identity of [1-^{14}C]- *and* [3-^{14}C]-*dihydroxyacetone*

Preparation of [α-^{14}C]*dihydroxyacetone.* L-Erythrulose (100 μmoles) was incubated at 37° for 2 hr. with [^{14}C]formaldehyde (50 μmoles, 4·0 μc) and yeast transketolase (0·4 ml.) in 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (100 ml., 0·2 M, pH 7·6), $MgCl_2$ (50 μmoles) and thiamine pyrophosphate (0·5 mg.; cf. Dickens & Williamson, 1958; for details see Batt *et al.* 1960*b*). In 120 min. 33 μmoles of formaldehyde had been utilized (acetylacetone analysis on 0·2 ml. portions). Ice-cold 20% perchloric acid (0·5 ml.) was added, the small precipitate of protein was removed and the supernatant was deionized with Bio-Deminrolit G. The filtrate and washings were evaporated to dryness *in vacuo*, the residue was redissolved in 1·0 ml. of water and chromatographed on paper as a band [solvent: butan-2-one–acetic acid–aqueous 4% (w/v) boric acid (9:1:1)]. The position of the dihydroxyacetone band was detected by marginal markers, the band was cut from the paper and eluted with water, the eluate was evaporated *in vacuo* to dryness and the evaporation repeated several times with additions of methanol to remove boric acid. Finally the aqueous solution of [α-^{14}C]dihydroxyacetone (10 μmoles) was made to 1·0 ml.

Enzymic phosphorylation of [α-^{14}C]*dihydroxyacetone and reduction to phosphoglycerol.* Glycerokinase was prepared from rat liver (Bublitz & Kennedy, 1954). The solution of labelled dihydroxyacetone (10 μmoles in 1·0 ml.) was transferred to a Warburg vessel with glycerokinase (2·0 ml.), $MgCl_2$ (20 μmoles), $NaHCO_3$ (100 μmoles), sodium

adenosine triphosphate (Sigma Chemical Co., Mo., U.S.A.; 60 μmoles) and water (to 6·0 ml.). Incubation at 37° under N_2+CO_2 (95:5, v/v) yielded CO_2 equivalent to 9·0 μmoles of dihydroxyacetone phosphate formation. After deproteinization at 0° with 20% perchloric acid (0·2 ml.), centrifuging, cautious neutralization of the supernatant with 20% KOH and removal of perchlorate by centrifuging, the final solution contained dihydroxyacetone phosphate. This was reduced by addition of α-glycerophosphate dehydrogenase (C. F. Boehringer und Söhne, Mannheim, Germany; 0·08 ml.), DPNH (0·9 ml., 0·5%) in tris buffer (1·3 ml., 0·2M, pH 7·6). The photometrically determined DPNH oxidation corresponded to the formation of 4·8 μmoles of α-glycerophosphate. Unlabelled α-glycerophosphate (560 μmoles) was then added, and the mixture was heated at 100° for 3 min., when 2·6 ml. of 50% (w/v) barium acetate was added to the cooled solution. The precipitate was removed, the supernatant and washings were adjusted to pH 9·0 by means of saturated $Ba(OH)_2$ and 4 vol. of ethanol were added. After being kept at 0° for 24 hr. the precipitate was collected, washed and dried (136 mg.; ester P content equiv.: 300 μmoles of barium α-glycerophosphate). This was fractionated by absorption on a column of Dowex-1 (formate form; 1 cm. × 18 cm.), washed with water (20 ml.) and eluted with 0·1M-ammonium formate, with collection of 10 ml. fractions. The fractions between 300 and 400 ml. of total eluate contained the single peak of α-glycerophosphate, which was again precipitated as barium salt from the concentrated eluate.

Degradation of the [^{14}C]*glycerophosphate.* Barium α-glycerophosphate (13·0 mg., prepared as described above) was dissolved in water (2·5 ml.) with a few drops of N-HCl. The barium was removed by sodium sulphate (0·12 ml., 0·5M) and unlabelled sodium α-glycerophosphate (to a total of 120 μmoles) was added: total volume was 10·0 ml. After ^{14}C determination by persulphate oxidation of a small sample, sodium periodate (0·6 ml., 0·5M) was added and then 2N-H_2SO_4 to pH 3. After keeping the solution for 1 hr. at room temperature, sodium sulphite (1 ml., 2%) was added. After a further 10 min. the solution was passed through an Amberlite IRA-400 (OH^- form) column (1 cm. × 10 cm.) and washed through with water to 50 ml. total eluate, a small portion of which was analysed for formaldehyde (acetylacetone method: 120 μmoles) and the formaldehyde in the whole was converted into the dimedone derivative for determination of radioactivity (43% of total radioactivity of original solution). The glycolaldehyde 2-phosphate, the other product formed from α-glycerophosphate by periodate oxidation, was eluted from the well-washed column by 20 ml. of 2N-H_2SO_4 (117 μmoles of total phosphate). After neutralization to pH 7·5, alkaline phosphatase (Sigma Chemical Co.; 20 mg.) was added, followed by incubation at 37° for 2 hr. Repetition of the periodate treatment on the hydrolysate gave 92 μmoles of formaldehyde, and the formaldehyde dimedone was prepared from the distillate of the neutralized solution. The second steam-distillate after acidification gave formic acid, which was converted into CO_2 by $HgCl_2$–acetate oxidation and converted into $BaCO_3$ for determination of radioactivity (cf. Batt *et al.* 1960*b*).

The results showed the following percentage of total radioactivity in the named carbon atoms of glycerol in α-glycerophosphate: C-1, 52%; C-2, 5%; C-3, 43%. It is clear from this result that both primary alcohol groups of dihydroxyacetone are approximately equally labelled, indicating that there is no appreciable enzymic discrimination between these two groups either by the transketolase reaction itself or by the kinase which phosphorylates the dihydroxyacetone formed by the transketolase. In accordance with theoretical expectations, therefore, dihydroxyacetone, unlike glycerol, behaves as a symmetrical molecule in these enzymic reactions.

DISCUSSION

Metabolism of L-*erythrulose in the intact rat*

The results summarized in Table 2 show clearly that ^{14}C from both C-1 and C-4 of the L-erythrulose molecule is fairly extensively utilized in the rat. Elimination in the urine is considerable, about one-third to one-sixth of the urinary excretion being due to a reduction of the ketotetrose to the corresponding tetritol, a threitol being formed which has been distinguished electrophoretically from erythritol, and is presumed to be L-threitol produced by the action of the DPN-xylitol (D-xylulose) dehydrogenase on L-erythrulose (Hollmann & Touster, 1957; Hickman & Ashwell, 1959; Hollmann, 1959).

Apart from the urinary excretion, extensive oxidation to respiratory carbon dioxide occurs, though to a different extent for individual carbon atoms, being more than 50% higher for C-1 of erythrulose than for C-4. This finding indicates that oxidation does not follow the complete conversion into a symmetrical molecule, such as reduction to threitol, before oxidation. Moreover, the time course of production of carbon dioxide is different for the two labelled erythruloses. Again, incorporation into liver glycogen is quite high for both labelled erythruloses, but higher for the ^{14}C-4- than for the ^{14}C-1-labelled compound, i.e. in the opposite sense to the evolution of carbon dioxide from these two carbon atoms.

Table 5. *Distribution of* ^{14}C *in glucose isolated from liver slices incubated with* L-[1-^{14}C]- *or* L-[4-^{14}C]-*erythrulose or* D-[4-^{14}C]*erythrose*

The percentage activity in the specified carbon atoms of the glucose molecule are given.

(*a*) Degradation by *Leuconostoc mesenteroides*

Rat no.	Substrate	C-1	C-2	C-3	C-4	C-5	C-6
8	L-[1-^{14}C]Erythrulose (duplicate runs)	42 47	0 0	0 0	8 12	2 4	48 37
14	L-[4-^{14}C]Erythrulose	23	3	9	29	4	37

(*b*) Degradation by *Lactobacillus casei*

		C-1 + C-6	C-2 + C-5	C-3 + C-4
21	L-[1-^{14}C]Erythrulose	88	1	11

(*c*) Periodate oxidation of glucosazone

		C-1 + C-2 + C-3	C-4 + C-5	C-6	Total ^{14}C recovered (%)
10	L-[1-^{14}C]Erythrulose	62	6	32	97
11		69	6	25	80
12		72	7	21	84
13		62	5	33	88
Average		66	6	28	88
10	L-[4-^{14}C]Erythrulose	25	18	57	88
11		34	16	50	100
12		42	17	41	98
13		40	3	57	85
Average		35	13	52	93
16	D-[4-^{14}C]Erythrose	22	5	73	84

Incorporation of isotope into the glucose carbon atoms of liver glycogen (Table 3) shows that with the [4-^{14}C]erythrulose labelling is probably symmetrical and mainly in C-1 and C-6 of glucose, but with [1-^{14}C]erythrulose it appears mainly in C-1 and much less in C-6. Labelling in C-4 plus C-5 of glucose is at a low level, suggesting that incorporation of respiratory $^{14}CO_2$, which is well known to enter in positions C-3 and C-4, is not an important feature of erythrulose metabolism. These results on glucose formation are in excellent general agreement with those found for liver slices, as far as the different experimental conditions permit.

Metabolism of L-*erythrulose and* D-*erythrose in rat-liver slices.* Comparison of the results given in Tables 2 and 4 and 3 and 5 shows such a close similarity for the metabolism of L-erythrulose in the intact rat and in rat-liver slices respectively that the liver may well be the main site of ketotetrose metabolism in the body. In the shorter (90 min.) experiments with liver slices, less percentage oxidation of both C-1 and C-4 of erythrulose to carbon dioxide occurred than in the 180 min. experiments in the whole rat, but nevertheless the proportions of oxidation at C-1 relative to C-4 show the same excess of oxidation at C-1 over C-4. The incorporation into glucose by the liver tissue of fed rats (used for the tissue-slice experiments) was in extent about the same as the incorporation (in fasting rats) into liver glycogen observed in the intact animals. However, while the average C-4 incorporation into glucose was greater than C-1 incorporation in the whole rat, the reverse was true in the rat-liver slices.

As in the intact rat, reduction of a part (average 12%) of the labelled erythrulose to tetritol also occurred, even under the aerobic conditions of the slice experiments, and unchanged erythrulose was recovered from the medium in amount about equal to that so reduced.

Localization of isotope from the two labelled erythruloses in the glucose formed by the tissue slices was almost the same as the localization in liver glycogen in the intact rat (Tables 3 and 5), the main difference being the slightly lower C-1 to C-3 glucose labelling with [4-^{14}C]erythrulose in rat-liver slices compared with that in the whole animal. Again, this might be connected with the use of fed rats for slices and fasted rats for experiments on the whole animal.

Degradation of glucose formed by the slices was more detailed (Table 5) than that of liver glycogen and included differentiation of labelling among C-1, C-2 and C-3 (by *Leuconostoc mesenteroides*), showing that most of the labelling was in C-1 with much lower amounts, or none, in C-2 and C-3. The peculiar and possibly erroneous results for C-4 of glucose by this method have already been discussed. *Lactobacillus casei* fermentation (Table 5) gave results in good agreement with those for

L. mesenteroides, showing 88% of total ^{14}C in C-1 plus C-6. Taking the results as a whole, this figure was fairly consistently obtained for combined radioactivity of the two terminal glucose carbons, whether [1-^{14}C]- or [4-^{14}C]-erythrulose was the ketotetrose administered: the main difference being due to the preponderance of 1-^{14}C of erythrulose C-1 of glucose, whereas 4-^{14}C of erythrulose enters mainly C-6 of the glucose molecule. Possible explanations of this finding are discussed below.

Tables 4 and 5 show some preliminary results obtained when D-[4-^{14}C]erythrose was incubated with rat-liver slices. The preponderance of labelling introduced into C-6 of glucose is even higher than with [4-^{14}C]erythrulose as substrate, being more than three times as high as in C-1+C-2+C-3 of glucose combined.

The tetritols are poorly metabolized by liver slices according to these experiments (Table 4), and erythritol appears not to be utilized. However, L-threitol does appear to enter the metabolic pathways leading to glucose as well as those giving carbon dioxide. One route for this would be the preliminary dehydrogenation of L-threitol to L-erythrulose by means of the DPN-xylitol (D-xylulose) dehydrogenase and DPNH. This reaction we have shown (Batt *et al.* 1960*b*) to give erythrulose almost equally labelled in C-1 and C-4 when terminally labelled threitol (i.e. 1-^{14}C or 4-^{14}C, both substances being therefore enzymically identical) is the substrate. Consequently L-[1-^{14}C]threitol should be metabolized after this dehydrogenation in exactly the same way as an equimolar mixture of [1-^{14}C]- and [4-^{14}C]-erythrulose. According to the results of Table 5, already discussed, this would be expected to give almost equal labelling of C-1 and C-6 of glucose. This possibility remains to be determined, however, owing to the much smaller incorporation and lesser availability of labelled threitol, the metabolism of which is on this hypothesis controlled by the rate of its enzymic dehydrogenation to erythrulose.

Some preliminary observations were made on the effect on the labelling of L-[1-^{14}C]- and L-[4-^{14}C]-erythrulose when these were incubated with rat-liver slices, with subsequent isolation and degradation of the erythrulose remaining, the general conditions being the same as in Table 4. Little exchange of isotope between C-1 and C-4 of erythrulose had occurred, showing that little of the residual erythrulose could have been reversibly equilibrated under these conditions with L-[1-^{14}C(4-^{14}C)]threitol. However, if, as is clear from our observations, the aerobic utilization of L-erythrulose is much more rapid than that of L-threitol, it is not necessarily to be expected that erythrulose entering the slices should exchange in this way, but it might be drained off more rapidly into other metabolic channels. Further work is needed on this point, e.g. under anaerobic conditions where oxidative utilization of erythrulose would not compete with the above-described exchange reaction.

Some possible routes of tetrose and tetritol metabolism. Fig. 1 summarizes the reactions already described at the beginning of this paper, or, in some cases (indicated by broken arrows) hypothetical reactions, which may be supposed to be concerned in the metabolism of L-erythrulose and L-threitol.

Of the known enzymic reactions, the action of transketolase on L-erythrulose might perhaps be expected to be of primary importance. This would yield 'active glycolaldehyde' together with an equimolar amount of glycolaldehyde (route *A*, Fig. 1). The metabolism of free glycolaldehyde in rats has been studied by Friedmann, Levin & Weinhouse (1956), who found that 2·25% of the dose of [2-^{14}C]glycolaldehyde was incorporated into liver glycogen, the distribution in glucose carbon atoms 1–6 being respectively 21, 26, 6, 7, 22 and 19%. Entry of glycolaldehyde (*B*, Fig. 1) was considered to be indirect via its transformation into glycine and serine. In our experiments [2-^{14}C]glycolaldehyde would arise from transketolase action upon [4-^{14}C]erythrulose. However, the low incorporation into C-2, C-3, C-4 and C-5 observed by us with this compound suggests that this route can play only a minor part in the metabolism of L-erythrulose in rat liver. The same conclusion can be drawn from the behaviour of the 'active glycolaldehyde' moiety (*C*, Fig. 1). Hiatt (1957) found that D-[1-^{14}C]ribose was converted into liver glycogen in the mouse by a transketolase pathway about as effectively as D-glucose, the labelling being mainly at C-1 (40%) and C-3 (45%) of the glucose molecule. Transketolase action on L-[1-^{14}C]erythrulose would be expected to give a similar distribution. However, in our experiments, in addition to labelling at C-1 (44%), low or zero labelling at C-3 and fairly heavy labelling (28%) at C-6 were observed, and this again appears to exclude a transketolase route of metabolism of L-erythrulose. It may be added that transketolase also appeared to play only a minor part in the metabolism of hydroxypyruvate in rat-liver tissue (Dickens & Williamson, 1959).

The other known metabolic reaction of free erythrulose is its reversible reduction to L-threitol (*D*, Fig. 1). The possible role of this in metabolism of L-threitol via erythrulose has already been discussed, and also the possibility that this enzymic exchange might cause double-ended labelling of erythrulose, as indicated on Fig. 1 by the double-labelling of the (symmetrical) L-threitol so produced (cf. Batt *et al.* 1960*b*). The resulting

L-[1:4-$^{14}C_2$]erythrulose would be expected to contribute isotope to all those carbon atoms denoted in Fig. 1 by * and °, the symbols used for 1-^{14}C and 4-^{14}C respectively. Incubation of liver slices with [1-^{14}C]- and [4-^{14}C]-erythrulose did not, however, cause much randomization between C-1 and C-4 of the residual erythrulose, as has already been mentioned.

We are therefore compelled to look for other and as yet unknown pathways of erythrulose metabolism. Of several possible routes the most probable include (*E*, Fig. 1) phosphorylation of L-erythrulose to give the 1-phosphate; a reaction closely resembling the action of fructokinase and ATP on fructose, but not in fact brought about by that enzyme with L-erythrulose as substrate (Batt *et al.* 1960*b*). L-Erythrulose 1-phosphate is split by an aldolase reaction (*H*, Fig. 1) to give reversibly dihydroxyacetone phosphate and formaldehyde; this reaction has already been discussed (see also Batt *et al.* 1960*b*), but whereas we have shown above that dihydroxyacetone behaves isotopically as expected, i.e. symmetrically, C-1 and C-3 being identical groups, this does not apply to dihydroxy-

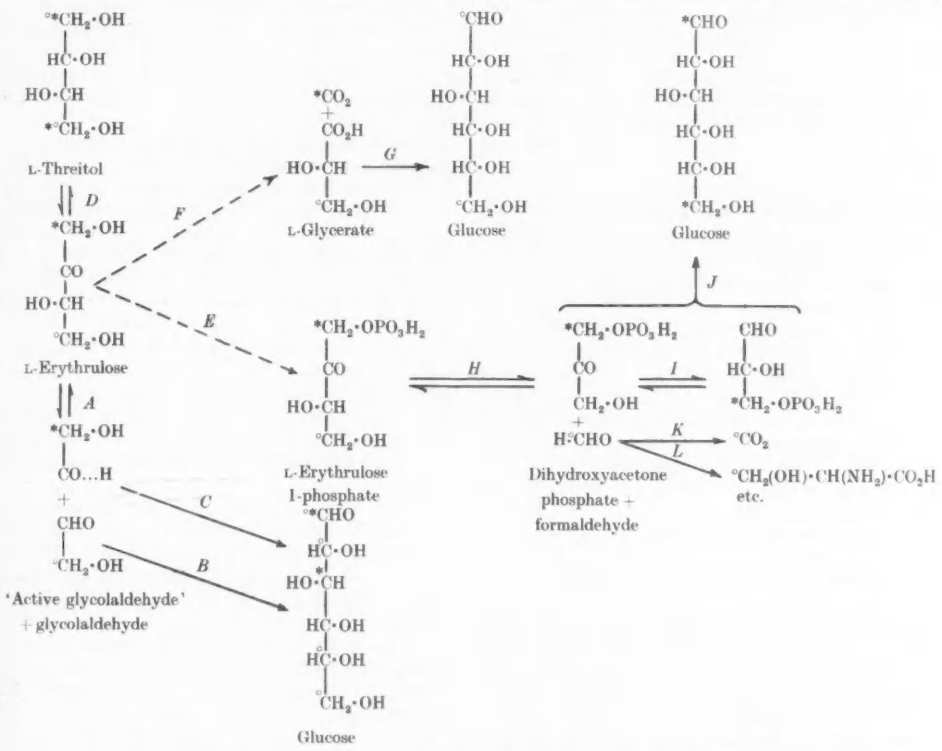


Fig. 1. Some possible metabolic pathways for specifically labelled tetroses and L-threitol. The following enzymic pathways of L-erythrulose are shown: *A*. Transketolase (with thiamine pyrophosphate as coenzyme) cleavage. *B*. [2-^{14}C]Glycolaldehyde incorporation into liver glycogen (Friedmann, Levin & Weinhouse, 1956) by an unknown route. *C*. Transketolase and transaldolase incorporation of CH_2·OH-labelled 'active glycolaldehyde' into liver glycogen (Hiatt, 1957). *D*. DPNH reduction of L-erythrulose by the DPN-xylitol (D-xylulose) dehydrogenase of liver. Reversibility causes randomization of erythrulose C-1 and C-4 labelling. *E*. Hypothetical phosphorylation with ATP plus a kinase. *F*. Hypothetical C-1 oxidation and decarboxylation to (non-natural) L-glyceric acid. *G*. Incorporation of L-[3-^{14}C]glycerate into liver glycogen (F. Dickens & D. H. Williamson, unpublished results). *H*. Phosphoketotetrose aldolase (Charalampous & Mueller, 1953; Charalampous, 1954) or aldolase from liver or muscle (Peanasky & Lardy, 1958). *I*. Triose phosphate isomerase. *J*. Aldolase and reversal of glycolysis. *K*. Formaldehyde dehydrogenase (Strittmatter & Ball, 1955). *L*. Serine aldolase (serine transhydroxymethylase; Alexander & Greenberg, 1955). * and °, 1-^{14}C- and 4-^{14}C-labelled atoms respectively (see text).

acetone phosphate, in which the labelling at C-1 as shown in Fig. 1 is therefore preserved from that originally present in [1-^{14}C]erythrulose 1-phosphate. If free dihydroxyacetone were an intermediate this would not apply, and glucose formed by way of the phosphorylation of the free substance should therefore present approximately equal labelling in C-1, C-3, C-4 and C-6, which was not observed.

Fig. 1 shows in outline the isomerization (*I*) of the triose phosphates and their condensation by aldolase to give fructose 1:6-diphosphate, which would yield (*J*) glucose equally labelled in C-1 and C-6 if equilibration by the triose phosphate isomerase (*I*) were complete; otherwise a preponderance of isotope at C-1 of glucose over that at C-6 would be expected. However, in fed animals particularly, dilution of the labelled glyceraldehyde phosphate produced by reaction *I* with tissue triose may lower the extent of labelling in C-6 of glucose by this route.

The formaldehyde (derived from C-4 of erythrulose) produced in reaction *H* can be oxidized (*K*) via formate to carbon dioxide or by combination with tetrahydrofolic acid can give hydroxymethyltetrahydrofolic acid, the well-known source of 'active one-carbon transfer' (*L*) for formation of C-3 of serine and incorporation into purines etc. Formate carbon enters the glucose molecule by this route predominantly at C-1 and C-6 of glucose (Bernstein, 1953). By this aldolase route therefore C-4 of erythrulose would only enter the glucose molecule indirectly, C-1 would do so predominantly at positions 1 and 6 of glucose, and only C-4 of erythrulose would be converted into carbon dioxide, via formaldehyde oxidation.

The experimental findings both in the intact rat and in liver slices show that C-1 of erythrulose is the main carbon atom metabolized to carbon dioxide, which is not in accord with the above-mentioned pathway being the principal one. Moreover, the expected approximately equal labelling at C-1 and C-6 of the glucose formed from L-[1-^{14}C]erythrulose, and much lower expected glucose labelling from the 4-^{14}C-labelled compound, are not consistent with the observed percentage incorporation or with the distribution of radioactivity in the glucose carbon atoms (Tables 2–5).

The remaining possible route included in Fig. 1 (*F*) for the incorporation of L-erythrulose follows the postulated removal of C-1 of erythrulose by oxidation and decarboxylation of the resulting keto acid. This would yield $^{14}CO_2$ only from 1-^{14}C of erythrulose, which, in fact, gave much higher yields than [4-^{14}C]erythrulose both in the whole animal and in liver slices (Tables 2 and 4). However, if this were the only route, no labelling, other than minor amounts at C-3 and C-4 of glucose due to carbon dioxide fixation, should have entered the glucose when [1-^{14}C]erythrulose was the substrate, and this was not so. The other product besides carbon dioxide from C-1 oxidation of erythrulose is expected to be L-glyceric acid (the 3-^{14}C-labelled acid from [4-^{14}C]erythrulose as substrate). F. Dickens & D. H. Williamson (unpublished work) have found that both D- and L-[3-^{14}C]glycerate are incorporated into rat-liver glycogen (or glucose when slices are used) to about the same extent and with similar labelling of glucose (at C-1 and C-6). Consequently, L-glycerate could well be an intermediate as shown (*G*) in Fig. 1. By this route [4-^{14}C]erythrulose would give glucose labelled at C-1 and C-6, though the expected equal labelling at each of these carbon atoms was not fully realized in the present experiments, there being considerable variations with the different experimental conditions.

Thus it is probable that several different pathways of metabolism for L-erythrulose exist simultaneously in the rat liver. One of these is the reversible reduction (*D*, Fig. 1) to L-threitol, which, as already described, could lead to partial or even complete randomization of ^{14}C between C-1 and C-4 of the erythrulose, whichever labelled form of erythrulose was the starting material. The probability that L-threitol itself is metabolized in liver by its primary conversion by this dehydrogenase into L-erythrulose has already been discussed. Evidently the extent of this randomization would greatly tend to complicate the interpretation and, if complete, should even render the metabolism of C-1- and C-4-labelled erythrulose the same, which was not so.

Finally, the metabolism of D-[4-^{14}C]erythrose (not included in Fig. 1) would be expected to begin with its transketolase conversion to give D-[6-^{14}C]-glucose 6-phosphate, which should yield glucose and glycogen labelled exclusively in the C-6 position. Although 73% of the labelling was in fact at C-6, the mode of entry of nearly all the remaining radioactivity into positions C-1–C-3 of glucose remains to be studied, by means of D-erythrose labelled on carbon atoms other than C-4.

SUMMARY

1. The metabolism of a number of specifically ^{14}C-labelled tetroses and tetritols has been studied in the intact rat and in rat-liver slices aerobically incubated.

2. In the intact fasted rat, intraperitoneally administered specifically labelled L-erythrulose is fairly extensively metabolized. Average recoveries of ^{14}C during 3 hr. after the injection were, from the 1-^{14}C-labelled compound, 25% in respiratory carbon dioxide, 3·8% in liver glycogen and

15% in urinary excretion. From the 4-^{14}C-labelled compound the corresponding values were 15·5, 4·8 and 20% respectively.

3. The time course of $^{14}CO_2$ elimination in respiration during the 3 hr. experiments differs for the two labelled erythruloses.

4. The glucose of the liver glycogen is differently labelled according to the labelled erythrulose given. With [1-^{14}C]erythrulose 62% of total labelling appeared in C-1+C-2+C-3 of glucose and 27% in C-6. With the 4-^{14}C-labelled compound a more symmetrical labelling (48 and 45% respectively) was observed in these groups of glucose carbon atoms. Minor amounts (about 10%) were found in C-4+C-5 of glucose.

5. Rat-liver slices also metabolize L-erythrulose, an average of only 13% of the substrate being recovered after incubation for 90 min. Of the remainder, $^{14}CO_2$ formation accounted for 12% of 1-^{14}C and 5% of 4-^{14}C of the original erythrulose activity, glucose contained 4·3 and 2·7% respectively, and reduction of part of the erythrulose to tetritol accounted for 12% with either labelled erythrulose.

6. Degradation of the glucose produced by rat-liver slices incubated with labelled L-erythrulose showed that the pattern of labelling was essentially similar to that in the whole animal, except for a slight preponderance in C-6 over C-1+C-2+C-3 when [4-^{14}C]erythrulose was the substrate. More elaborate degradations were done on the glucose produced by liver slices, and showed that with both labelled ketotetroses nearly all the labelling reported for C-1+C-2+C-3 of glucose is in fact in C-1.

7. The urinary metabolites included about 4% of the dose of ^{14}C as tetritols, threitol being the only detectable substance in this fraction of the excretion products; no erythritol was formed. The nature of the tetritols produced in the slice experiments was not studied, but it is probable that in both instances reduction of L-erythrulose to L-threitol had occurred by means of the diphosphopyridine nucleotide-xylitol (D-xylulose) dehydrogenase of mammalian liver.

8. D-[4-^{14}C]Erythrose is metabolized by rat-liver slices, yielding in 90 min. 1% of the labelling as $^{14}CO_2$ and about 2·5% as glucose. Here the labelling of the glucose carbon atoms is predominantly (73%) in C-6 and 22% in C-1+C-2+C-3 with only 5% in C-4+C-5.

9. The metabolism of some labelled tetritols in rat-liver slices has been studied. [1-^{14}C]Erythritol, electrophoretically separated from the product of catalytic hydrogenation of L-[4-^{14}C]erythrulose, was not appreciably converted into $^{14}CO_2$ or [^{14}C]-glucose. Various preparations of L-threitols, produced from L-[1-^{14}C]- and L-[4-^{14}C]-erythrulose, were metabolized, though to a less extent than L-erythrulose itself. It is suggested that preliminary enzymic oxidation to L-erythrulose would be consistent with the extent of L-threitol metabolism observed.

10. Possible pathways of tetrose metabolism are discussed. It was shown in connexion with the incorporation into glucose that the two $CH_2 \cdot OH$ groups of free dihydroxyacetone behave, as expected, identically in enzymic reactions, unlike the corresponding groups in glycerol.

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Endogenous Sulphate Acceptors in Rat Liver

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There is now abundant evidence to show that adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1957) is an active form of sulphate from which the sulphate group can be transferred to various acceptors under the influence of specific sulphokinases to form sulphate esters or sulphamino compounds (Lipmann, 1958; Gregory & Robbins, 1960). In the presence of added adenosine triphosphate, Mg^{2+} and SO_4^{2-} ions, yeast preparations synthesize adenosine 3′-phosphate 5′-sulphatophosphate by a two-stage reaction. The first stage involves the reversible action of an adenosine triphosphate-sulphurylase to give adenosine 5′-sulphatophosphate and pyrophosphate and this is followed by non-reversible phosphorylation of adenosine 5′-sulphatophosphate by adenosine triphosphate in the presence of a specific adenosine 5′-sulphatophosphate-kinase to give adenosine 3′-phosphate 5′-sulphatophosphate (Bandurski, Wilson & Squires, 1956). The synthesis of adenosine 3′-phosphate 5′-sulphatophosphate by the soluble material of the cytoplasm of mammalian liver is also known to be a two-stage process, and although an unequivocal proof is not yet available it seems reasonably certain that the steps are the same as those in the yeast preparations (Robbins & Lipmann, 1958).

On incubation of particle-free high-speed supernatants of iso-osmotic-potassium chloride homogenates of rat liver with adenosine triphosphate, SO_4^{2-} and Mg^{2+} ions, it was observed that the rate of formation of adenosine 3′-phosphate 5′-sulphatophosphate diminished with increasing length of incubation and, eventually, after reaching a maximum concentration, the overall amount of adenosine 3′-phosphate 5′-sulphatophosphate decreased. Some of the diminution in the rate of formation of the compound might be attributable to the accumulation of pyrophosphate, which would tend to reverse the first stage of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1958) and actual loss of the compound would be expected owing to the action of a specific 3′-nucleotidase (Brunngraber, 1958). With $^{35}SO_4^{2-}$ ions, followed by chromatography and radioautography of the incubates after various times, it has been shown that a further contributing factor to disappearance of adenosine 3′-phosphate 5′-sulphatophosphate is the transfer of sulphate from the compound to endogenous acceptors to

give sulphate esters which are revealed as radioactive spots on the radioautographs. The evidence for this is now presented and the nature of the acceptors is discussed. A preliminary communication has appeared (Spencer, 1959*a*).

EXPERIMENTAL

Liver preparations. Livers from M.R.C. hooded-strain rats were removed immediately after the animals were killed and were homogenized in 10 vol. of iso-osmotic KCl (11·4 g./l.) at 2° with a glass homogenizer. The homogenate was centrifuged in a Spinco preparative ultracentrifuge at 54 000 *g* (rotor no. 30) for 90 min. After removal of the superficial fatty layer, the supernatant was distributed amongst a number of tubes of 1 ml. capacity and stored at −20°.

Reaction mixtures. The standard reaction mixture was 40 μl. of liver preparation plus 10 μl. of a solution that had been adjusted to pH 7·4 with NaOH and which contained 1 μmole of adenosine triphosphate (ATP), 1 μmole of KH_2PO_4 and 0·3 μmole of $MgSO_4$. In the radioactive-tracer experiments 0·3 μmole of $MgCl_2$ was substituted for the $MgSO_4$ and 5 μc of $Na_2{}^{35}SO_4$ (code SJSI, specific activity greater than 100 mc/mg. of S; The Radiochemical Centre, Amersham, Bucks.) was added. Incubation was at 38° for various time intervals and the enzymic reaction was stopped and the proteins were precipitated by heating the mixture in a boiling-water bath for 2 min. The mixture was then centrifuged.

Chromatography. The supernatant after centrifuging of the deproteinized reaction mixture was applied to Whatman no. 1 paper in amounts varying from 5 to 25 μl. but usually 10 μl. Three solvent systems were used: acetone–water–acetic acid (90:9:1, by vol.) for 3–4 hr., butanol–acetic acid–water (50:12:25, by vol.) for 16 hr. and *iso*butyric acid–aq. 0·3N-NH_3 soln., (5:3, v/v) for 16 hr. (cf. Suzuki & Strominger, 1959). The R_F values of $SO_4{}^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′-[^{35}S]-sulphatophosphate were all 0·0 in the acetone solvent, 0·08, 0·04 and 0·06 respectively in the butanol solvent and 0·20, 0·26 and 0·34 in the *iso*butyric acid solvent. After air-drying, radioautographs were prepared by placing the papers in contact with Ilford Industrial B X-ray film for 5–10 days. Alternatively, chromatograms were scanned with a C 100 Actigraph automatic chromatogram strip scanner (Nuclear-Chicago Corp., Ill., U.S.A.) at a speed of 6 in./hr. and with a $\frac{1}{8}$ in. slit. Occasionally the radioactivity of the spots was measured (G.M. mica end-window) after elution of the spots with water and drying the eluates on stainless-steel planchets (5 cm.^{2}). Corrections were made for dead time of the instrument and background but no correction for self-absorption was made and the values obtained were considered only relative.

Preparation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate. A yeast extract containing an active adenosine 3′-phosphate 5′-sulphatophosphate-synthesizing system was prepared by the method of Nose & Lipmann (1958). The extract (2 ml.), plus 0·5 ml. of a solution containing 50 μmoles of ATP, 50 μmoles of KH_2PO_4 and 250 μc of $Na_2{}^{35}SO_4$, which had been adjusted to pH 7·4 with NaOH, was incubated for 1 hr. at 38° and then heated in a boiling-water bath for 2 min. After cooling and centrifuging the mixture, the total supernatant was placed on Whatman no. 17 paper, 20 cm. wide, and subjected to electrophoresis at 80 v for 16 hr. at 2° with 0·1 M-ammonium acetate–acetic acid, pH 5·6. Under these conditions the $^{35}SO_4{}^{2-}$ ions had run 30 cm. and the strong radioactive band approximately midway between the origin and the $^{35}SO_4{}^{2-}$ ions was eluted with water and the eluate was freeze-dried. The dried material was dissolved in 1 ml. of water and applied as a band on Whatman no. 3 MM paper. The chromatogram was developed for 4 hr. with acetone–water (9:1). Strong radioactivity was shown only at the origin and this was eluted with 1 ml. of water; the eluate was divided into 100 μl. portions and stored at −20°. On chromatography of this material in a number of different solvents, all the radioactivity, apart from a trace of $^{35}SO_4{}^{2-}$ ions, was found in the adenosine 3′-phosphate 5′-sulphatophosphate spot. No ultraviolet-absorbing spots other than adenosine 3′-phosphate 5′-sulphatophosphate were observed. The amount of the compound varied in different preparations from 0·3 to 1·0 μmole/ml.

Adenosine 5′[^{35}S]-sulphatophosphate of low specific radioactivity was a gift from Dr N. Ringertz. Adenosine 5′[^{35}S]-sulphatophosphate with a specific activity of 7000 counts/sec./mg. was prepared by the method of Reichard & Ringertz (1959).

Estimation of adenosine 3′-phosphate 5′-sulphatophosphate. Use was made of the method of Robbins & Lipmann (1957) in which the formation of *p*-nitrophenyl sulphate by transfer of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate to *p*-nitrophenol under the influence of phenolsulphokinase is estimated from the decrease in absorption by the *p*-nitrophenol anion at 400 mμ. Fresh, crude rat-liver KCl-homogenate supernatant was used as a source of phenolsulphokinase and ethylenediaminetetra-acetate (EDTA) was added to suppress formation of adenosine 3′-phosphate 5′-sulphatophosphate (Brunngraber, 1958). The EDTA also partially inhibited the specific 3′-nucleotidase of rat liver (Brunngraber, 1958) which degrades adenosine 3′-phosphate 5′-sulphatophosphate (Fig. 2). As will be shown, crude liver preparations contain endogenous sulphate acceptors but when an added acceptor is present in high concentration only a very small proportion of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate is transferred to the endogenous acceptors. Under the conditions employed at least 98% of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate was transferred to *p*-nitrophenol.

To 10 μl. of the adenosine 3′-phosphate 5′-sulphatophosphate-containing solution was added 10 μl. of liver preparation, 5 μl. of *p*-nitrophenol solution at pH 7·4 (15 μm-moles of *p*-nitrophenol) and 10 μl. of 0·04 M-EDTA containing 0·025 M-cysteine at pH 7·4. After incubation for 90 min. at 38° the mixture was deproteinized with 200 μl. of ethanol and centrifuged. A 100 μl. portion of the supernatant was diluted with 50 μl. of 0·1N-NaOH and the extinction at 400 mμ measured with the Hilger Uvispek spectrophotometer and micro-cell attachment. A blank determination, in which 5 μl. of water was substituted for the *p*-nitrophenol solution, and a control, in which the *p*-nitrophenol solution was added immediately before deproteinization, were also carried out. The difference between the control and blank readings at 400 mμ represents 15 μm-moles of *p*-nitrophenol and the difference between this extinction and that between the test and

blank readings represents, proportionally, the amount of *p*-nitrophenol which has been sulphated, and thus the amount of adenosine 3′-phosphate 5′-sulphatophosphate.

Extraction of phenols from rat liver. An extract of liver containing phenols and from which neutral and basic compounds were eliminated was prepared in the following manner. The supernatant (10 ml.) of the KCl homogenate of rat-liver was made 0·1 N with respect to HCl, extracted with ether (3 × 10 ml.) and the combined ether extracts were washed with 0·1 N-NaOH (2 × 5 ml.). The aqueous alkaline solution was washed with ether (2 × 10 ml.), then made 0·1 N with respect to HCl and extracted with ether (3 × 10 ml.). The combined ether extracts were washed once with 5 ml. of water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The extract, which was dissolved in 0·5 ml. of water, gave strong positive $FeCl_3$, 2:6-dichloroquinonechloroimide and Millon's tests for phenols.

Enzymic hydrolysis of the sulphated endogenous acceptors. The arylsulphatase of *Aspergillus nidulans* (C.M.I. 16643) was a preparation used by Spencer (1959*b*). Crude digestive juice of *Helix pomatia* was collected from the crops of freshly dissected snails. A purified preparation of the arylsulphatase of the digestive gland of *H. pomatia* was obtained from Dr G. M. Powell.

The enzyme preparation (10 μl.) plus 10 μl. of M-sodium acetate–acetic acid buffer, pH 6·0, was incubated at 38° for 4 hr. with 20 μl. of the incubated liver preparation. The mixture was then heated at 100° for 2 min., cooled and 20 μl. was applied to paper for chromatography.

Since PO_4^{3-} ion is a potent inhibitor of many sulphatases the incubated liver preparation was made with the standard reaction mixture incorporating $Na_2{}^{35}SO_4$ and adjusted to pH 7·4 but without the KH_2PO_4. After incubation for 2 hr. at 38° the mixture was heated at 100° for 2 min., cooled and centrifuged. The radioautograph pattern of the ester sulphates in the supernatant, as shown by chromatography with all three of the standard chromatographic solvents, was the normal pattern and in every way th ame as when KH_2PO_4 was present in the incubated reaction mixture.

Sulphation of added acceptors. To a mixture of 30 μl. of the liver preparation and 10 μl. of a solution at pH 7·4 containing 1 μmole of ATP, 1 μmole of KH_2PO_4, 0·3 μmole of $MgCl_2$ and 5 μc of $Na_2{}^{35}SO_4$, was added 10 μl. of a solution of the acceptor. After incubation for 1 hr. the mixture was heated in a boiling-water bath for 2 min., cooled and centrifuged and 10 μl. of the supernatant was spotted on Whatman no. 1 paper for chromatography.

The acceptors were dissolved in water or in 0·05 M-acid or -alkali, the pH was adjusted to 7·4 with HCl or NaOH and the resulting solution or suspension was added to the incubation mixture. Steroids were dissolved in ethanol and the requisite amounts of the solutions were added to empty tubes. The solvent was evaporated off and 10 μl. of water was added, followed by the liver preparation and solution containing ATP etc.

Collection of urine and bile. The 24 hr. urine from rats that had each received an intraperitoneal injection of 500 μc of $Na_2{}^{35}SO_4$ in NaCl was collected by placing the rats in large Büchner funnels. Bile was collected from bile cannulae inserted in rats which had been anaesthetized firstly with ether and then with 30 mg. of Nembutal injected subcutaneously. A 500 μc dose of $Na_2{}^{35}SO_4$ in NaCl was injected into the right jugular vein. Bile was collected for 3 hr. after injection of the radioactive compound.

RESULTS

Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate. At 38° in the presence of added ATP, Mg^{2+}, PO_4^{3-} and SO_4^{2-} ions the particle-free rat-liver preparation synthesized adenosine 3′-phosphate 5′-sulphatophosphate at a rate which diminished with time. The concentration of adenosine 3′-phosphate 5′-sulphatophosphate in the system was maximum at 90 min. and thereafter decreased until, after 6 hr. incubation, the amount remaining was negligible (Fig. 1). The highest concentration attained was 20 μM and since the added ATP concentration was 0·02 M and that of the SO_4^{2-} ions was 6 mM, neither of these reactants was rate-limiting, at least in the initial stages.

Breakdown of adenosine 3′-phosphate 5′-sulphatophosphate. As well as the slowing down of the rate of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate in the liver system it was evident that breakdown of the compound had taken place. This was investigated separately by incubating the ^{35}S-labelled compound with a rat-liver supernatant (which had been stored at −20° for 3 months) in the presence of Mg^{2+} and PO_4^{3-} ions. Portions of the incubation mixture were removed after various times, boiled for 2 min., centrifuged and the supernatant was examined chromatographically with the *iso*butyric acid–aq. NH_3 soln. solvent. The chromatograms were scanned with the Actigraph automatic strip scanner and the amounts of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, adenosine 5′[^{35}S]-sulphatophosphate and $^{35}SO_4^{2-}$ ions were assessed by measuring the areas under the curve. A more accurate estimation of the $^{35}SO_4^{2-}$

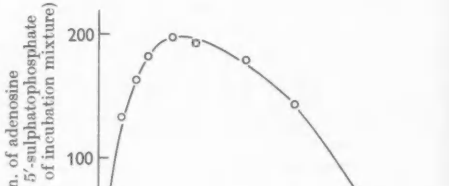

Fig. 1. Accumulation of adenosine 3′-phosphate 5′-sulphatophosphate by the particle-free supernatant of an iso-osmotic-KCl rat-liver homogenate at 38° and pH 7·3 in the presence of 0·02 M-ATP, 0·02 M-KH_2PO_4 and 6 mM-$MgSO_4$.

ions produced was obtained by elution of the $^{35}SO_4^{2-}$ ions from the chromatograms and measurement of the radioactivity by a plating technique. After 1·5 hr. all the adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate had disappeared (Fig. 2), but only approx. 95% of the initial amount of ^{35}S could be accounted for as $^{35}SO_4^{2-}$ ions. A further amount of SO_4^{2-} ions, equal to approx. 4% of the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate introduced, was recovered after hydrolysis of the 1·5 hr. incubation mixture with N-HCl for 1 hr. No breakdown of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate occurred in the control which contained boiled liver preparation.

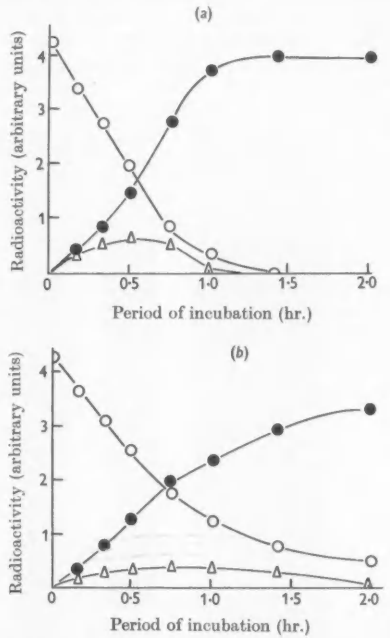

Fig. 2. Degradation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate by the rat-liver supernatant which had been stored at −20° for 3 months. (*a*) Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (0·1 mM) was incubated at 38° in the presence of 0·02 M-KH_2PO_4 and 6 mM-KH_2PO_4 at pH 7·3. After the required period a portion of the incubation mixture was heated at 100° for 2 min., cooled, centrifuged and 10 μl. of the supernatant was chromatographed on Whatman no. 1 paper for 16 hr. with *iso*butyric acid–aq. 0·3 N-NH_3 soln. (5:3). Radioactivity of the spots corresponding to $^{35}SO_4^{2-}$ ions, adenosine 5′[^{35}S]-sulphatophosphate and adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was assessed by measuring the area under the curve obtained by scanning the chromatograms with a C 100 Actigraph automatic strip scanner. The amount of radioactivity is expressed in arbitrary units. (*b*) Conditions were the same as in (*a*) but EDTA was incorporated at a concentration in the incubation mixture of 0·08 M. ○, Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate; ●, $^{35}SO_4^{2-}$ ion; △, adenosine 5′[^{35}S]-sulphatophosphate.

These results suggested that at least two mechanisms were responsible for breakdown of adenosine 3′-phosphate 5′-sulphatophosphate, the major one leading to the formation of inorganic sulphate and the other to a new form or forms of 'bound' sulphate. The formation of inorganic sulphate is readily explained as being the result of the action of the specific 3′-nucleotidase (Brunngraber, 1958) or phosphatase (Robbins & Lipmann, 1958) which is known to be present in rat liver, followed by a splitting of the phosphosulphate link of the adenosine 5′-sulphatophosphate so formed (cf. Reichard & Ringertz, 1959). In the chromatograms of the liver–adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate experiments a radioactive spot with the same R_F as synthetic adenosine 5′-sulphatophosphate appeared after incubation for 10 min. The concentration of this spot rose during the incubation but eventually it all disappeared (Fig. 2). Its identity with synthetic adenosine 5′-sulphatophosphate was confirmed by radioautography after paper electrophoresis under the conditions used for the preparation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (see Experimental section). The breakdown of adenosine 5′-sulphatophosphate to give inorganic sulphate was shown by incubation of 0·1 μmole of the synthetic ^{35}S-labelled compound in 10 μl. of water with 30 μl. of the liver preparation and 10 μl. of water containing 0·3 μmole of Mg^{2+} ions and 1 μmole of PO_4^{3-} ions. After chromatography with the *iso*butyric acid–aq. NH_3 soln. solvent the presence of $^{35}SO_4^{2-}$ ions was demonstrated by radioautography. Measurements of the radioactivity of the $^{35}SO_4^{2-}$ ions and adenosine 5′[^{35}S]-sulphatophosphate spots by elution and plating showed that the major fate of the sulphate of adenosine 5′-sulphatophosphate in the system used (i.e. in the absence of added ATP) was to be split off as SO_4^{2-} ions, all of the ^{35}S-labelled compound being hydrolysed in 2·5 hr. On examination under u.v. light (253 mμ) all the ^{35}S-labelled compound had disappeared after 2·5 hr. and new u.v.-absorbing spots with R_F values corresponding to adenylic acid (R_F 0·45) and adenosine (R_F 0·50) were seen. Approx. 1% of the ^{35}S of the adenosine 5′[^{35}S]-sulphatophosphate was incorporated into a new spot at R_F 0·45 (*iso*butyric acid–aq. NH_3 soln.).

When 0·08 M-EDTA was incorporated into the incubation mixtures containing rat-liver supernatant which had been stored at −20° for 3 months, the rate of breakdown of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-

sulphatophosphate was inhibited (Fig. 2). With the latter compound the non-specific 5′-nucleotidase activity at pH 7·3 was completely inhibited (cf. Spencer, 1958), and apart from residual adenosine 5′[^{35}S]-sulphatophosphate the only u.v.-absorbing spot observed was adenylic acid. With fresh liver preparations the breakdown of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was so rapid that adenosine 5′[^{35}S]-sulphatophosphate was not seen on chromatograms of the liver–adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate incubation mixtures. In the presence of 0·08 M-EDTA the fresh liver preparations did produce adenosine 5′[^{35}S]-sulphatophosphate.

'Bound' sulphate. Radiochromatographs of the standard radioactive reaction mixture after different incubation times revealed the presence of a number of radioactive spots with R_F greater than that of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate (Fig. 3). The concentration of these unknown spots increased with the length of time of incubation of the reaction mixture while the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate concentration declined (cf. Fig. 1).

With the *iso*butyric acid–aq. NH_3 soln. solvent, $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, adenosine 5′[^{35}S]-sulphatophosphate and up to seven other distinct radioactive spots were observed (Table 1 and Fig. 3). With butanol–acetic acid–water solvent, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate were sometimes obscured by the strong $^{35}SO_4^{2-}$ ion spot at R_F 0·08, but eight other radioactive spots were observed. With a band of the incubation mixture and the acetone–acetic acid–water solvent, 15 radioactive bands were seen, apart from $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate, all of which remained at the origin. Many of the bands on the one-way chromatograms were not single entities. Elution of the single band showing the highest R_F in acetone–acetic acid–water followed by rechromatography with *iso*butyric acid–aq. NH_3 soln. revealed the presence of five different radioactive spots. A two-way chromatogram with the acetone and *iso*butyric acid solvents showed 15 radioactive spots apart from $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate. In the two-dimensional chromatogram many of the weak radioactive components previously observed when the acetone–acetic acid–water solvent was used alone were not seen. Thus the total number of radioactive compounds in the incubated material must be greater than 15. However, most of the work to be reported was of an exploratory nature and was carried out by one-way chromatography. The *iso*butyric acid–aq. NH_3 soln. solvent was preferred since it distinguished $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate and this solvent has been used throughout unless otherwise stated.

The most feasible suggestion about the nature of the unknown spots was that they were sulphate esters formed by transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to endogenous acceptors in the rat-liver supernatant. The evidence to support this suggestion was as follows.

After prolonged dialysis (24 hr. against six changes of distilled water) the rat-liver supernatant

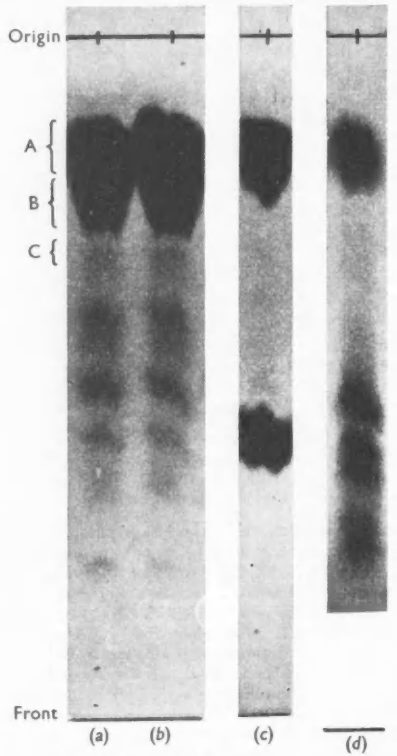

Fig. 3. Radioautographs of *iso*butyric acid–aq. NH_3 soln. chromatograms. (*a*) Standard incubation mixture incorporating $Na_2{}^{35}SO_4$ after 1 hr. incubation at 38°. (*b*) As (*a*), with the incorporation of 0·01 M-hydroxyproline. (*c*) As (*a*), with the incorporation of 0·01 M-*p*-cresol. (*d*) Bile collected from a rat which had been injected with 500 μc of $Na_2{}^{35}SO_4$. A, $^{35}SO_4^{2-}$ ions; B, adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate; C, adenosine 5′[^{35}S]-sulphatophosphate.

could still produce adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate under the standard-incubation conditions, but the unknown radioactive spots were either missing on the chromatograms or very weak in comparison with those given by the undialysed preparation. Addition of the supernatant of boiled fresh rat-liver KCl supernatant to the dialysed preparation followed by incubation under the standard conditions resulted in the appearance of all the unknown spots.

Hydrolysis of the incubated standard radioactive reaction mixture with 0·1 N-HCl for 15 min. at 100° before chromatography resulted in the disappearance of the majority of radioactive spots, including the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate. The spots that remained were very much weaker than the corresponding ones on the chromatograms of the unhydrolysed liver incubates. After hydrolysis with N-HCl for 30 min. at 100° only $^{35}SO_4^{2-}$ ions were detected on the chromatograms. Most sulphate esters are known to be hydrolysed under these conditions.

In the experiment in which adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was incubated with the liver preparation approximately 4% of the added compound was converted into the new forms of 'bound' sulphate. Radioautographs of the incubation mixture showed the 'bound' sulphate as barely discernible radioactive spots, which were, however, distinct from $^{35}SO_4^{2-}$ ions, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate. The experiment was repeated with mM-adenosine 5′-[^{35}S]-sulphatophosphate in the incubation mixture, i.e. about ten times the amount used previously, and the mixture after incubation for 1 hr. was examined chromatographically in the three solvent systems. The radioautographs prepared with an exposure time of 3 weeks showed all the radioactive spots previously observed in the radioautographs of the incubation mixture containing liver preparation, ATP, PO_4^{3-}, Mg^{2+} and $^{35}SO_4^{2-}$ ions, i.e. when adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was being continuously formed.

With the standard reaction mixture the particle-free supernatants prepared from 10% (w/v) homogenates in iso-osmotic KCl (11·4 g./l.) of rat kidney, spleen, lung, heart and testis were all able to produce adenosine 3′-phosphate 5′-sulphatophosphate. The number of sulphate acceptors in each of the tissue preparations, as revealed by the radio-

Table 1. *R_F values of radioactive spots on chromatograms of the incubated standard reaction mixture*

Standard reaction mixture, incorporating $Na_2{}^{35}SO_4$, was incubated for 4 hr. at 38°, then heated at 100° for 2 min., centrifuged and 10 µl. samples of the supernatant were chromatographed in three different solvent systems. The radioactive spots were localized by radioautography. The experiment was repeated with the iso-osmotic KCl (11·4 g./l.) supernatants of other rat tissues instead of liver. Male rats were used throughout.

*iso*Butyric acid–aq. NH_3 soln.				Acetone–water–acetic acid		Butanol–acetic acid–water	
Liver	Kidney	Spleen	Assignment	Liver	Assignment	Liver	Assignment
0·18	0·18	0·18	$^{35}SO_4^{2-}$	0·00	$^{35}SO_4^{2-}$, adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate, adenosine 5′[^{35}S]-sulphatophosphate	(0·04)	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate
						(0·06)	Adenosine 5′[^{35}S]-sulphatophosphate
0·24	0·24	0·24	Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate	0·04	—	0·08	$^{35}SO_4^{2-}$
0·31	0·31	—	Adenosine 5′[^{35}S]-sulphatophosphate	0·07	—	0·27	—
0·39	0·39	—	—	0·09	—	0·37	—
0·45	—	—	—	0·11	—	0·42	Steroid sulphates?
0·53	0·53	0·53	—	0·14	—	0·46	Steroid sulphates?
0·61	0·61	0·61	Arylsulphates?	0·15	—	0·53	—
0·69	0·69	0·69	—	0·21	—	0·60	Arylsulphates?
0·74	0·74	—	—	0·29	—	0·73	—
0·79	0·79	0·79	Steroid sulphates and arylsulphates?	0·39	—	0·80–0·88	—
				0·46	—		
				0·56	—		
				0·64	—		
				0·74	Arylsulphates?		
				0·84	Arylsulphates?		
				0·93	Steroid sulphates?		

active spots on the chromatogram, was in all cases very much less than in the liver preparations. The spots which were observed were all common with those of the liver preparation but less intense. Formation of adenosine 3′-phosphate 5′-sulphatophosphate and sulphate transfer to endogenous acceptors has also been shown for sheep-trachea epithelium (D. K. Watkins, unpublished observation).

Nature of the endogenous sulphate acceptors. Some of the endogenous acceptors of normal liver might have been derived directly from the food supply or from the blood present in the liver. Supernatants were therefore prepared from the iso-osmotic-KCl (11·4 g./l.) homogenates of livers from rats that had been starved for 48 hr., but given water without restriction, and from livers that had been perfused with cold iso-osmotic KCl. These preparations were incubated for 4 hr. in the standard reaction mixture. In each case the patterns of radioactive spots on the chromatograms prepared with all three standard solvents were the same as those derived from normal liver.

Evidence accumulated which suggested that some of the endogenous acceptors were phenols. The ether extract of rat liver (10 μl.) was incubated for 15 min. at 38° with 50 μl. of the standard reaction mixture containing $Na_2{}^{35}SO_4$, then it was heated at 100° for 2 min., centrifuged and 10 μl. of the supernatant was applied for chromatography. The radioautographs showed that some radioactive spots, in particular those with R_F 0·61 in *iso*butyric acid–aq. NH_3 soln., R_F 0·84 in acetone–water–acetic acid and R_F 0·60 in butanol–acetic acid–water, were greatly increased in intensity compared with those given under the same conditions in the absence of the extract. Some of these intensified spots were presumably arylsulphates formed from the phenols present in the ether extract.

A portion (2 ml.) of the standard reaction mixture that had been incubated for 4 hr. was chromatographed as a band on Whatman no. 3MM paper with the acetone–water–acetic acid solvent. The five radioactive bands with the highest R_F values, i.e. 0·93, 0·84, 0·74, 0·64 and 0·55, were eluted with 0·5 ml. of water and their ultraviolet-absorption spectra were examined. The spectra in each case showed a peak or inflexion in the 280 mμ region and high absorption at lower wavelengths, although this was somewhat masked by non-specific end-absorption. Spectra of this type are characteristic of aromatic compounds.

After hydrolysis of the standard reaction mixture which had been incubated for 2 hr. with the aryl-sulphatase preparations from *A. nidulans* and *H. pomatia*, chromatography followed by radioautography showed that certain of the radioactive spots were considerably diminished in intensity. With the acetone–water–acetic acid solvent this applied particularly to the fast-moving spots and in the *iso*butyric acid–aq. NH_3 soln. solvent the spot R_F 0·61 was greatly affected. Adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′-[^{35}S]-sulphatophosphate were unaffected by the enzymes and none of the other spots completely disappeared. After hydrolysis with the crude digestive juice of *H. pomatia*, known to contain aryl-, glyco-sulphatase and steroid sulphatase, the majority of the radioactive spots completely disappeared. Those remaining (R_F 0·79, 0·74 and 0·31 in *iso*butyric acid–aq. NH_3 soln.) were greatly diminished in intensity. The adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate spot had also disappeared and a new radioactive spot (R_F 0·0 in butanol–acetic acid–water; R_F 0·12 in *iso*butyric acid–aq. NH_3 soln; R_F 0·0 in acetone–water–acetic acid) of similar intensity appeared. Further work, which will be reported elsewhere, has shown that a transfer of $^{35}SO_4{}^{2-}$ ions from the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate of the liver incubation mixture to an endogenous acceptor present in the snail juice had taken place under the influence of a specific sulphokinase in the juice.

Sulphation of known compounds by the rat-liver preparation. Another method of approach towards the identification of the unknown spots was made by attempting to recognize the classes of compounds that could be sulphated by the liver preparation. The procedure was to incubate possible acceptors with the standard reaction mixture for 1 hr., followed by chromatography and radioautography of the protein-free supernatant of the boiled reaction mixture that had been incubated. The radioautographic pattern was then compared with that obtained in the absence of added acceptor. When transfer of $^{35}SO_4{}^{2-}$ ions from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to an added acceptor took place, little or no adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate remained; the sulphated endogenous acceptors were either completely absent or very weak and the presence of the sulphated acceptor was shown as a strong new radioactive spot or spots (Fig. 3*c*). When no such transfer took place, substantial amounts of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate were present and the endogenous ester sulphate pattern (Fig. 3*b*) was the same as that of the standard reaction mixture (Fig. 3*a*). In initial experiments the steroids were dissolved in propane-1:2-diol (Nose & Lipmann, 1958) and diluted with an equal volume of water. After incubation of each of the steroids with the liver preparation four radioactive spots were observed on the chromatograms developed with butanol–acetic acid–water. Three of the spots were found to be formed from

Table 2. *R_F values of new radioactive spots on chromatograms of the standard reaction mixture incubated with various acceptors for 1 hr.*

R_F values in parentheses indicate that the spot was faint relative to the other spots.

Acceptor	Concn. of acceptor in incubation mixture (M)	R_F		
		*iso*Butyric acid–aq. NH_3 soln.	Acetone–water–acetic acid	Butanol–acetic acid–water
Phenols				
Phenol	0·01	0·53	0·86	—
o-Cresol	0·01	0·60	0·85	0·60
m-Cresol	0·01	0·60	0·85	0·60
p-Cresol	0·01	0·60	0·85	0·60
m-Hydroxybenzoic acid	0·01	0·36	0·39	0·51
p-Hydroxybenzoic acid	0·01	0·48	0·46	—
Thymol	0·01	—	0·79	—
L-Adrenaline	0·01	0·38, 0·41, 0·50	—	—
Tyramine	0·01	0·38, 0·47, 0·50	0·07, 0·58, 0·72	0·37, (0·45), 0·49
Steroids				
Androsterone	0·01	0·79	0·93	0·45
Dehydro*iso*androsterone	0·01	0·79	0·93	0·45
Pregnenolone	0·01	0·79	—	0·45
Oestrone	0·01	0·79	—	0·45
Oestradiol	0·01	0·79	—	0·45
Alcohols				
Ethanol	0·68	0·35	—	0·45
Butanol	0·40	—	—	0·73
Propane-1:3-diol	0·31	—	—	0·37, (0·46), 0·73
Propane-1:2-diol	0·31	0·30, 0·51, 0·62	—	0·54, 0·64, 0·85
Aromatic mines				
α-Naphthylamine	0·01	0·59	—	(0·45), 0·71
β-Naphthylamine	0·01	—	—	(0·45), 0·88

propane-1:2-diol in the absence of the steroid (Table 2). In subsequent experiments the steroids were treated as described in the Experimental section.

Table 2 lists the acceptors added and the R_F values of the new radioactive spots detected on the chromatograms. That the sulphation of the added acceptors occurred through the transfer of $^{35}SO_4^{2-}$ ions from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was shown by repetition of the experiments but with substitution of 10 μl. of the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate solution for the ATP–KH_2PO_4–$MgCl_2$–$Na_2{}^{35}SO_4$ mixture. The radioautographs in all cases showed the same new spots as those found when the liver preparation was allowed to produce the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate *in situ*. The following compounds did not appear to be sulphated when incorporated into the standard reaction mixture at a final concentration of 0·01 M: bilirubin, cholic acid, L-threonine, L-serine, L-hydroxyproline, L-hydroxyglutamic acid and choline chloride. Rat bile diluted with 4 vol. in the incubation mixture also failed to give new radioactive spots.

Rat urine and bile after injection of $Na_2{}^{35}SO_4$. More than 95% of the radioactivity of the 24 hr. urine sample collected from rats that had been injected with 500 μc of $Na_2{}^{35}SO_4$ was present as $^{35}SO_4^{2-}$ ions. On chromatography with the *iso*butyric acid–aq. NH_3 soln. solvent, the pattern of radioactive spots was very similar to that previously observed with the incubation mixtures with liver (Tables 1 and 3). One spot (R_F 0·48), which did not occur in the liver pattern, was seen in the urines from both male and female rats and an additional spot (R_F 0·51) was seen only in the radiochromatograph of urine from males. The spots at R_F 0·79 and 0·74 were very weak in the urine from males compared with that from females. In the acetone–water–acetic acid solvent, distinctions between the chromatograms of both urines were seen, particularly amongst the spots with low R_F values (Table 3). Several spots which were present in the liver pattern were not found in the radiochromatographs of urine from rats of either sex. All the radioactive spots seen on radioautographs of *iso*butyric acid–aq. NH_3 soln. chromatograms of urine disappeared after hydrolysis of the urines with N-HCl for 30 min. at 100° or with crude snail juice at pH 5·5 for 4 hr. and only $^{35}SO_4^{2-}$ ions remained.

About 50% of the radioactivity of the bile from a male rat which had been injected with $Na_2{}^{35}SO_4$

could be attributed to $^{35}SO_4^{2-}$ ions, although the percentage varied slightly with the time of collection after the injection. Four radioactive spots, R_F 0·27, 0·33, 0·48 and 0·56, which were not previously observed in the liver pattern, were seen on radiochromatographs with *iso*butyric acid–aq. NH_3 soln. of the bile whereas the spots (R_F 0·31 and 0·61) of the liver pattern were not present in the bile (Table 3). Although there were slight changes in the relative intensities of the radioactive spots in the radiochromatographs of bile collected at 15 min. intervals, the overall pattern remained constant up to 3 hr. after the injection of $Na_2{}^{35}SO_4$. Similar, but fewer, radioactive spots have been observed in rabbit bile (Boström & Vestermark, 1959). After hydrolysis of the bile with N-HCl for 30 min. at 100° chromatography showed that at least 95% of radioactivity was present as $^{35}SO_4^{2-}$ ions. The radioactivity remaining was spread throughout the paper and no definite spots were seen. The same result was obtained by hydrolysis with the crude digestive juice of *Helix pomatia*. These results would seem to preclude the possibility that the radioactivity of any of the unknown spots was due to [^{35}S]taurine. Taurine formation from inorganic sulphate in liver after injection of $^{35}SO_4^{2-}$ ions has been noted (Boström & Aqvist, 1952).

DISCUSSION

The accumulation of adenosine 3′-phosphate 5′-sulphatophosphate by particle-free supernatants of iso-osmotic-potassium chloride homogenates of rat liver in the presence of ATP, Mg^{2+} and SO_4^{2-} ions (Fig. 1) was found to be dependent not only on the rate of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate but also on the rate of breakdown. The mechanism of synthesis of adenosine 3′-phosphate 5′-sulphatophosphate has been established (Robbins & Lipmann, 1957) but apart from brief notes to the effect that rat liver contains a phosphatase capable of 3′-dephosphorylating adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1958; Brunngraber, 1958) little has been reported about degradation of adenosine 3′-phosphate 5′-sulphatophosphate.

When adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was incubated with the particle-free liver preparations in the absence of added ATP and Mg^{2+} ions the major fate of the sulphate group of the nucleotide was to be split off as inorganic sulphate. One mechanism of $^{35}SO_4^{2-}$ ion formation in this system was shown to involve 3′-dephosphorylation of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to give adenosine 5′-sulphatophosphate followed by cleavage of the phosphate–sulphate linkage to give adenylic acid and $^{35}SO_4^{2-}$ ions. Both the enzymes concerned were inhibited by EDTA. It is possible that the enzyme which desulphates adenosine 5′-sulphatophosphate can also desulphate adenosine 3′-phosphate 5′-sulphatophosphate to yield SO_4^{2-} ions and adenosine 3′:5′-diphosphate, which could then be further degraded by 3′-dephosphorylation. This latter reaction is known to take place in liver extracts (Brunngraber, 1958) but proof of the overall reaction mechanism would depend on the identification of adenosine 3′:5′-diphosphate as an inter-

Table 3. *R_F values of radioactive spots on chromatograms of the urine of male and female rats and the bile of a male rat after injection with* $Na_2{}^{35}SO_4$

Each rat received 500 μc of $Na^{35}SO_4$ in 1 ml. of 0·9% sodium chloride soln.

*iso*Butyric acid–aq. NH_3 soln.				Acetone–water–acetic acid		
Liver	Urine of male	Urine of female	Bile	Liver	Urine of male	Urine of female
0·18	0·18	0·18	0·18	0·00	0·00	0·00
0·24	—	—	—	0·04	—	—
—	—	—	0·27	0·07	—	—
0·31	—	—	—	—	0·08	—
—	—	—	0·33	0·09	0·09	0·09
0·39	0·39	0·39	0·39	—	0·10	—
0·45	0·45	0·45	0·45	0·11	—	0·11
—	0·48	0·48	0·48	0·14	—	0·14
—	0·51	—	—	0·15	—	—
0·53	0·53	0·53	—	—	0·17	—
—	—	—	0·56	0·21	0·21	0·21
0·61	0·61	0·61	—	0·29	0·29	0·29
0·69	—	0·69	0·69	0·39	0·39	0·39
0·74	0·74	0·74	0·74	0·46	0·46	0·46
0·79	0·79	0·79	0·79	0·56	0·56	0·56
				0·64	0·64	0·64
				0·74	0·74	0·74
				0·84	0·84	0·84
				0·93	0·93	0·93

mediate product. No evidence can be offered on this point since the crude liver extract used in the present investigation produced some adenosine 3′:5′-diphosphate when SO_4^{2-} ion was transferred from adenosine 3′-phosphate 5′-sulphatophosphate to endogenous sulphate acceptors. A further reaction that was considered was the formation of SO_4^{2-} ions and ATP from adenosine 5′-sulphatophosphate and pyrophosphate under the influence of the ATP-sulphurylase which is present in the rat-liver supernatant. However, the liver preparation also contained an active pyrophosphatase and it was therefore unlikely that the concentration of endogenous pyrophosphate was sufficient for the above-mentioned mechanism to contribute significantly to the formation of SO_4^{2-} ions from adenosine 5′-sulphatophosphate.

A further fate of adenosine 3′-phosphate 5′-sulphatophosphate when incubated with the liver preparation was the transfer of the sulphate group to endogenous acceptors. This transfer occurred to a slight but significant extent, which was probably limited by the small amounts of acceptors present. When adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was used in the experiment the sulphated compounds appeared on chromatograms as radioactive spots with R_F values greater than adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and adenosine 5′[^{35}S]-sulphatophosphate in the three solvent systems used. Identical spots were seen on chromatograms of liver incubates in which adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate was continuously formed from ATP and $^{35}SO_4^{2-}$ ions. Similar spots, but fewer in number, have been previously obtained in comparable circumstances (Vestermark & Boström, 1959*a*) and it is probable that these are also sulphate esters of endogenous acceptors.

It is known (Gregory & Robbins, 1960) that the particle-free rat-liver preparation contains specific enzymes which can transfer the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate to phenols, phenolic steroids, non-phenolic steroids, arylamines and mono- and di-hydric alcohols. The particle-free liver preparation used in the present investigation transferred sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to all of the established acceptors (Table 2) and it can therefore be expected that many of the unknown endogenous acceptors belong to these classes of compounds. By adding known sulphate acceptors to the liver system followed by chromatography of the liver incubates it has been possible, in certain cases, to apportion areas of the chromatograms to different types of sulphate esters. Thus in *iso*butyric acid–aq. ammonia solution the radioactive spots formed from phenols had R_F 0·48–0·60 and all the steroids had R_F 0·79 (Table 2). The radioactive spots formed from the endogenous acceptors with these R_F values may therefore contain aryl and steroid sulphates respectively. Further evidence for the aryl nature of the spot R_F 0·61 was obtained by u.v. spectroscopy and by hydrolysis with arylsulphatases.

Little further is known about the nature of the endogenous sulphate acceptors. It is significant that many of the acceptors and the enzymes responsible for their sulphation are common to a number of organs (Table 1). This would suggest that either the acceptors are formed in the individual organs by metabolic pathways that are common to all the organs or that they are derived from one particular source and then carried round the body by the blood. The presence, in radiochromatographs of urine and bile, of sulphated compounds with the same R_F values as the sulphated acceptors of liver would further suggest that many acceptors are end products of metabolism which are excreted as sulphate esters rather than as active intermediates. However, the possibility that some of sulphated liver acceptors are low-molecular-weight intermediates in the synthesis of sulphated mucopolysaccharides or that they are inert forms of physiologically active compounds (cf. Spencer, 1956) is deserving of further investigation.

The presence of new radioactive spots in chromatograms of liver incubates to which potential acceptors had been added has been taken as indicating the formation of sulphate esters of the acceptors. A partial confirmation of this supposition was obtained for all the compounds listed in Table 2 by showing that the same new radioactive spots were formed in the absence of ATP when added adenosine 3′-phosphate 5′-sulphatophosphate was used as the sulphate donor. Further, with phenol and *p*-nitrophenol the R_F values of the respective spots were the same as those of authentic specimens of phenyl sulphate and *p*-nitrophenyl sulphate. One pitfall in making this general assumption when crude liver extracts are being used is that the added compound or its metabolites may in some way potentiate the ability of an endogenous compound to accept sulphate, thus giving rise to a new spot. The potentiation of the synthesis of arylamine sulphate esters by steroid sulphates (Roy, 1960) is an example of the kind of reaction that might occur. For this reason particular care should be exercised in interpreting the presence of the two or more new radioactive spots; for example, the naphthylamines (Table 2). In some cases the added compound has sufficient sites which could be sulphated to allow multiple spots to be accounted for solely as sulphate esters of the added acceptor. Thus propane-1:2-diol gives three spots which could be the two isomeric monosulphates

and the disulphate, but such an explanation will not hold for propane-1:3-diol (Table 2). Tyramine and L-adrenaline each give three spots which could be any of the two isomeric ring *O*-sulphates, the *N*-sulphate and, with adrenaline, the side-chain *O*-sulphate, or combinations. Another consideration is that the added acceptor may be metabolized in some way before or after sulphation. For example, in particle-free liver extracts L-adrenaline is known to be *ortho*-methylated (Axelrod, 1957) under certain circumstances.

The failure of the liver extract to sulphate bilirubin is unexpected since bilirubin sulphate has been found in rat bile (Isselbacher & McCarthy, 1959). Choline sulphate, which is formed by fungi and algae (Spencer & Harada, 1959), was not synthesized by the liver extract. None of the hydroxyamino acids was sulphated at the concentration employed (0·01 M) but it should be pointed out that very little sulphation of the primary aliphatic alcohols was observed at this concentration (cf. Table 2 and Vestermark & Bostrom, 1959*b*). One point that should be considered in evaluating these negative results is that sulphation may occur but the sulphate ester is hydrolysed by a sulphatase in the liver extract. However, with choline it is known that mammalian tissues are unable to desulphate choline sulphate (Morimoto, 1937).

SUMMARY

1. In the presence of adenosine triphosphate, Mg^{2+} and SO_4^{2-} ions particle-free supernatants of iso-osmotic-potassium chloride homogenates of rat liver accumulated adenosine 3′-phosphate 5′-sulphatophosphate. The rate of accumulation of adenosine 3′-phosphate 5′-sulphatophosphate depended both on the rate of synthesis and the rate of breakdown.

2. In the absence of adenosine triphosphate the rat-liver preparation degraded adenosine 3′-phosphate 5′-sulphatophosphate to SO_4^{2-} ions and adenosine monophosphate and eventually adenosine. One mechanism of degradation involved 3′-dephosphorylation to give adenosine 5′-sulphatophosphate, which was then desulphated yielding SO_4^{2-} ions and adenosine monophosphate. Both stages of this reaction were inhibited by ethylenediaminetetra-acetic acid.

3. A further fate of the sulphate group of adenosine 3′-phosphate 5′-sulphatophosphate in the presence of the liver extract was to be transferred to endogenous acceptors with the formation of sulphate esters.

4. Extracts of kidney, spleen, heart, lung and testes produced adenosine 3′-phosphate 5′-sulphatophosphate and transferred sulphate from this compound to endogenous acceptors.

5. Evidence has been obtained which suggests that some of the endogenous acceptors are phenols and possibly steroids.

6. The radiochromatograph patterns of urine and bile from rats which had received injections of $Na_2{}^{35}SO_4$ were compared with the radiochromatographs of the sulphated endogenous acceptors of liver. Some common radioactive spots were observed.

7. On incubation of various potential sulphate acceptors with the liver extract in the presence of adenosine triphosphate, Mg^{2+} and $^{35}SO_4^{2-}$ ions new radioactive spots were observed on chromatograms. Evidence suggesting that these spots were the sulphate esters of the acceptors has been presented but pitfalls in the interpretation of the chromatograms have been pointed out.

8. In this system sulphate esters were formed from all the tested phenols, steroids, alcohols and arylamines. Tyramine, adrenaline and propane-1:2-diol each gave three new radioactive spots. Bilirubin, cholic acid, L-threonine, L-serine, L-hydroxyproline; L-hydroxyglutamic acid and choline chloride did not appear to be sulphated.

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The Role of Choline Sulphate in the Sulphur Metabolism of Fungi

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The sulphate ester, choline sulphate, is somewhat of a biological curiosity. It was first isolated by Woolley & Peterson (1937) from *Aspergillus sydowi* in a yield greater than 0·2% of the dry weight of the mycelium, and similar high concentrations were later found in *Penicillium chrysogenum* (De Flines, 1955; Stevens & Vohra, 1955), in lichens of the *Rocella* species (Lindberg, 1955*a*) and the red alga *Gelida cartilaginum* (Lindberg, 1955*b*). Recent studies on the distribution of choline sulphate production (Harada & Spencer, 1960; Ballio *et al.* 1960) have shown a high degree of concurrence between the presence or absence of the compound and accepted taxonomic division. Synthesis of choline sulphate appeared to be limited to the higher fungi since it was not found in any Phycomycetes, and, of the Ascomycetes, the Endomycetales, including baker's yeast, did not accumulate this sulphate ester. Other Ascomycetes, including the Spaeriales, produced choline sulphate as also did all the Basidiomycetes and Fungi Imperfecti. One or two exceptions to this taxonomic division were noted, particularly amongst the Fungi Imperfecti, but these exceptions were in confirmation of previous morphological evidence which re-locates the fungi in classes which do not produce choline sulphate. None of the bacteria tested produced choline sulphate (Harada & Spencer, 1960).

The relatively large amounts of choline sulphate found in the fungal mycelia would suggest that the ester is an end product or by-product of sulphur or choline metabolism rather than an active intermediate; it could also be envisaged as a waste or storage product. However, the compound can be used as the sole source of sulphur for the growth of various fungi and also as a source of choline by the choline-less strain of *Neurospora crassa* (Stevens & Vohra, 1955; Harada & Spencer, 1960). Furthermore, choline sulphate and inorganic sulphate must possess some common metabolic pathway since mutant strains of *Aspergillus nidulans* and *Penicillium notatum* could utilize neither inorganic sulphate nor choline sulphate as sulphur sources but the parent strains grew readily on either compound (Hockenhull, 1948, 1949). Egami & Itahashi (1951) have shown that *Aspergillus oryzae* utilized choline sulphate for growth much more readily than inorganic sulphate, and competitive-metabolism experiments with the two sulphur sources (Itahashi, 1954) were interpreted as showing that choline sulphate was an intermediate in the utilization of the sulphur of inorganic sulphate. Similar experiments with *P. chrysogenum*, on the other hand, have shown that inorganic sulphate was used preferentially to choline sulphate for penicillin production (Stevens & Vohra, 1955). The evidence is thus somewhat controversial.

Harada & Spencer (1960) concluded that there were two possible ways in which choline sulphate could be connected with the utilization of the sulphur of inorganic sulphate for growth purposes by fungi; firstly, that the choline sulphate-producing and non-producing fungi metabolized sulphate in the same manner and that choline sulphate formation was just a side reaction in the former class of mould, and, secondly, that the two classes of fungi metabolize sulphate differently, choline sulphate formation being an obligatory stage only in the choline sulphate-producing fungi. It was the purpose of the present study to investigate these two alternatives.

As a first objective an attempt was made to find out why some fungi produced choline sulphate and others did not. Choline sulphate synthesis by *A. sydowi* is thought to involve the enzymically catalysed transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to choline (Kaji & McElroy, 1958). It is further known that formation of adenosine 3′-phosphate 5′-sulphatophosphate in yeast involves a reaction between adenosine triphosphate and SO_4^{2-} ions to give adenosine 5′-sulphatophosphate and pyrophosphate and that this is followed by 3′-phosphorylation of the adenosine 5′-sulphatophosphate to give adenosine 3′-phosphate 5′-sulphatophosphate (Robbins & Lipmann, 1957). There are several places in this overall scheme where choline sulphate production could be stopped. One possibility, the absence of choline, has already been ruled out by two different methods (Harada & Spencer, 1960; Ballio *et al.* 1960), and it is hardly likely that there is insufficiency of adenosine triphosphate. The other

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possibilities have therefore been examined. A preliminary communication has appeared (Spencer & Harada, 1959).

EXPERIMENTAL

Materials

$Na_2{}^{35}SO_4$ (code SJSI, specific activity greater than 100 mc/mg. of S) was obtained from The Radiochemical Centre, Amersham, Bucks. Adenosine 3′-phosphate 5′-[^{35}S]-sulphatophosphate was prepared by a biosynthetic method (Spencer, 1960). Choline [^{35}S]sulphate was synthesized from $H_2{}^{35}SO_4$ (The Radiochemical Centre) by the method of Schmidt & Wagner (1904), and had a specific activity of 14 000 counts/sec./mg. Adenosine 5′[^{35}S]-sulphatophosphate was synthesized by the method of Reichard & Ringertz (1959) and had a specific activity of 7000 counts/sec./mg.

The choline sulphate-producing fungi used were: *Aspergillus oryzae*, CMI: 17299; *Aspergillus nidulans*, CMI: 16643; *Aspergillus terreus*, LSH: Ab2; *Aspergillus sydowi*, CMI: 63904; *Penicillium aurantio-brunneum*, LSH: P2; *Penicillium notatum*, LSH: P45; *Penicillium chrysogenum*, CMI: 62728, *Penicillium citreo-roseum* LSH: P22.

The fungi not producing choline sulphate were: *Rhizopus stolonifer* (+), CMI: 57761; *Eremothecium ashbyii*, CMI: 31269; *Nematospora gossypii*, CMI: 31279; *Mucor hiemalis*, CMI: 21217; *Torulopsis utilis*, CMI: 23311; baker's yeast (Harada & Spencer, 1960).

Culture conditions. Fungi were grown at 27° either in static culture in 100 ml. of medium contained in 250 ml. conical flasks or shaking-culture in 200 ml. of medium in 1 l. flat-bottomed flasks. Vessels were inoculated with 0·5 ml. of spore suspension containing 10^7–10^8 spores/ml., prepared by washing spores from maltose–agar or glucose–peptone–yeast extract–agar with sterile water.

The following media were used: the Czapek-Dox liquid medium, containing 50 g. of glucose, 2·0 g. of $NaNO_3$, 0·4 g. of KH_2PO_4, 0·5 g. of KCl, 0·5 g. of $MgSO_4,7H_2O$ and 0·01 g. of $FeSO_4,7H_2O$ in 1 l. of water; a glucose–asparagine medium containing 10 g. of glucose, 1·5 g. of asparagine, 1·5 g. of $(NH_4)H_2PO_4$, 1·0 g. of KH_2PO_4, 0·5 g. of $MgSO_4$, 0·5 g. of NaCl, 20 mg. of inositol, 1 mg. of thiamine and 1 μg. of biotin in 1 l. of water; a glucose–peptone–yeast extract medium containing 20 g. of glucose, 10 g. of Bacto-peptone, 3 g. of Bacto-yeast extract and 0·6 g. of Na_2SO_4 in 1 l. of water. All media were adjusted to pH 5·4 with HCl or NaOH before sterilization for 20 min. at 15 lb./in.^{2}. Sulphate esters, apart from choline sulphate, which is stable under the sterilization conditions, were Seitz-filtered and added to sterilized media.

In experiments where the weight of the dried mycelium was measured, the mycelium was harvested by filtering through muslin, washed well with water and dried *in vacuo* over P_2O_5 for 2 days. The cells of some fungi, such as *E. ashbyii*, *T. utilis* and baker's yeast, were harvested by centrifuging.

Competitive metabolism. The complex glucose–asparagine medium was supplemented with normal or radioactive Na_2SO_4 or choline sulphate as shown in the legend to Fig. 1. The radioactive materials were diluted with the appropriate carrier compounds to give activities of the order of 94 counts/sec./ml. of medium for Na_2SO_4 and 112 counts/sec./ml. for choline sulphate.

The fungi were grown in 200 ml. of medium at 27° with shaking. At the commencement of the experiment, and thereafter every 24 hr., 5 ml. of the culture medium was aseptically removed, centrifuged to remove the mycelium, and 1 ml. of the supernatant was diluted to 5 ml. with water. A portion (0·8 ml.) of the diluted material was dried on stainless-steel planchets (5 cm.^{2}) and the radioactivity measured (G.M. mica end-window). Corrections were made for dead time of the instrument, background and decay.

After good growth had been observed (3, 5 or 6 days, Fig. 1) the mycelium was harvested, washed well with water and dried *in vacuo* over P_2O_5. A portion (200 mg.) of each mycelium was hydrolysed by heating with 20 ml. of 20% (w/v) HCl in 50% (w/v) formic acid at 90° for 5 hr. The hydrolysate was evaporated to dryness and the residue taken up in 10 ml. of 0·1 N-HCl. The solution was filtered and the precipitate washed with water. The filtrate and washings were made up to 50 ml. with water and the combined cystine and cysteine content was estimated by the method of Sullivan, Hess & Howard (1942). To 20 ml. of the solution was added 30 mg. of carrier cystine in 1 ml. of 0·02 N-HCl and the total cystine was then isolated in approximately 50% yield by the mercaptide method (Stekol, 1957). A portion (2·5 mg.) of the isolated cystine was weighed on to a planchet (5 cm.^{2}) and dissolved in 0·5 ml. of 0·1 N-NaOH. After drying the sample, the radioactivity was counted as before. No correction was made for self-absorption and the values in Table 2 should be considered only relative.

A preliminary experiment with *A. oryzae* and *A. nidulans* was carried out with Czapek-Dox medium and higher sulphur concentrations. The sulphur sources were $MgSO_4,7H_2O$ (200 mg. of $SO_4{}^{2-}$ ions/l.) with and without choline sulphate (200 mg. of $SO_4{}^{2-}$ ions/l.). $Na_2{}^{35}SO_4$ was incorporated into both media to give 220 counts/sec./ml. Samples of the culture media were removed and the radioactivity was assessed as before.

Acetone-dried fungal preparations. The fungus was grown on Czapek-Dox or glucose–peptone–yeast-extract media for 3–4 days with shaking. The mycelium was harvested, washed well with water and aerated in a large volume of tap water for 4 hr. (cf. Shepherd, 1956). After removal of as much water as possible by filtration, the mycelium was ground in a cooled mortar with ice-cold acetone and allowed to stand in acetone at 0° for 1 hr. The acetone was then quickly removed by filtration under suction and the precipitate washed well with acetone at 0°. All operations in which acetone was present were carried out in a cold room. The mycelium was finally dried *in vacuo* over P_2O_5 and stored at −20° in tightly stoppered bottles. For enzyme experiments a crude suspension of the dried material was made by homogenizing 25 mg. with 0·5 ml. of water in a small test tube and with a Teflon pestle. Occasionally an aqueous extract of the dried material was prepared by centrifuging the crude suspension and pouring off the supernatant.

Synthesis of choline sulphate in vitro. To 40 μl. of crude suspension of the acetone-dried preparation was added 10 μl. of a solution which had been adjusted to pH 7·3 with NaOH and which contained 1 μmole of adenosine triphosphate (ATP), 1 μmole of $PO_4{}^{2-}$ ion, 0·3 μmole of $MgCl_2$, 0·5 μmole of choline chloride and 5 μc of $Na_2{}^{35}SO_4$. After

incubation at 38° for 90 min. the mixture was placed in a boiling-water bath for 2 min., cooled and centrifuged. A portion (20 μl.) of the supernatant was applied to Whatman no. 1 paper and the chromatogram developed with acetone–water (9:1) for 3–4 hr. Radioautographs were prepared with Ilford Industrial B X-ray film and an exposure time of 7–10 days. A spot on the radioautograph at R_F 0·26 indicated the synthesis of choline sulphate (Harada & Spencer, 1960). Occasionally, choline sulphate formation was verified chromatographically with *iso*-butyric acid–aq. 0·3 N-NH_3 soln. (5:3) for 16 hr. (R_F 0·46). Relative assessment of the amount of choline sulphate formed in different systems was obtained by elution of the radioactive choline sulphate spot with water, drying the eluate on a planchet (5 cm.^{2}) and measuring the radioactivity as before.

Synthesis of adenosine 3′-phosphate 5′-sulphatophosphate in vitro. The incubation mixture was the same as that used for choline sulphate synthesis but choline chloride was omitted. After incubation for 1 hr. at 38° the mixture was heated at 100° for 2 min., cooled and centrifuged. The presence of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate in the supernatant was shown by paper electrophoresis as detailed below. None of the compound was formed in controls with boiled fungal preparations.

The supernatant (40 μl.) was applied to Whatman no. 3MM paper and subjected to electrophoresis for 16 hr. at 4 v/cm. in 0·1 M-ammonium acetate buffer, pH 5·6. The adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and authentic compound run simultaneously were revealed by radioautography as spots with approximately 60% of the mobility of $^{35}SO_4^{2-}$ ions. Occasionally, a faint spot of choline [^{35}S]sulphate was observed at the origin. This was presumably formed by transfer of $^{35}SO_4^{2-}$ ions from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to endogenous choline.

The presence of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate in the supernatant was confirmed by transfer of $^{35}SO_4^{2-}$ ions from the adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to *p*-nitrophenol under the influence of phenolsulphokinase. The source of phenolsulphokinase was a particle-free iso-osmotic KCl (11·4 g./l.) preparation of rat liver (Spencer, 1960) and 0·08 M-ethylenediaminetetra-acetic acid (EDTA) was incorporated to inhibit completely both stages of the adenosine 3′-phosphate 5′-sulphatophosphate-synthesizing system which is present in the preparation (Brunngraber, 1958). To 30 μl. of the supernatant was added 10 μl. of a *p*-nitrophenol solution at pH 7·3 containing 1 μmole of *p*-nitrophenol and 10 μl. of rat-liver preparation. After incubation for 1 hr., protein was denatured by heating at 100° for 2 min. and, after centrifuging, 20 μl. of the supernatant was placed on Whatman no. 1 paper and chromatographed. In the radioautographs a spot at R_F 0·85 (acetone–water) or 0·65 (*iso*butyric acid–aq. NH_3 soln.) indicated the formation of *p*-nitrophenyl sulphate and thus the presence of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate in the supernatant from the incubation of the fungal extract. No *p*-nitrophenyl sulphate was formed in controls in which the supernatant from the incubation of boiled fungal extract was used. The arylsulphatase which is present in most fungi and which is capable of hydrolysing *p*-nitrophenyl sulphate was destroyed when the fungal incubation mixture was boiled.

Choline sulphokinase. To 10 μl. of fungal preparation was added 10 μl. of adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate solution (approx. 50 μm-moles and 100 counts/sec.) and 10 μl. of a solution containing 0·5 μmole of choline chloride and 1 μmole of PO_4^{2-} ions adjusted to pH 7·3. Incubation was for 1 hr. at 38° and enzyme action was stopped by heating at 100° for 2 min. After centrifuging, the presence of choline [^{35}S]sulphate was shown by chromatography and radioautography. Boiled fungal extracts did not produce choline [^{35}S]sulphate under these conditions.

Table 1. *Synthesis of choline sulphate* in vitro *by* Aspergillus oryzae

To 25 μl. of crude aqueous suspension of acetone-dried *A. oryzae* was added 5 μl. of water and 10 μl. of a solution adjusted to pH 7·3 with NaOH and containing 1 μmole of ATP, 1 μmole of KH_2PO_4, 0·3 μmole of $MgCl_2$ and 5 μc of $Na_2{}^{35}SO_4$. After incubation at 38° for 90 min., the protein was denatured at 100° and 10 μl. of a clear supernatant obtained by centrifuging was chromatographed with the acetone–water (9:1) solvent. The choline sulphate spot at R_F 0·26 was eluted and the radioactivity of the eluate determined. A control in which the $Na_2{}^{35}SO_4$ was added immediately before heating at 100° was included. The various compounds added were dissolved in water, adjusted to pH 7·3 and substituted for the 5 μl. of water.

Incubation mixture	Choline [^{35}S]sulphate (counts/min.)
Complete	1940
Control	120
Minus ATP	180
Minus $MgCl_2$	105
Plus 0·01 M-choline chloride	3780
Plus 0·01 M-choline chloride but with the aqueous extract of the fungi	3220
Plus 0·01 M-choline chloride and 0·0125 M-cysteine hydrochloride	4430
Plus 0·01 M-choline chloride, M-cysteine hydrochloride and 0·08 M-EDTA but minus $MgCl_2$	150

RESULTS

The synthesis of choline [^{35}S]sulphate from $^{35}SO_4^{2-}$ ions by crude suspensions of the dried mycelia of *A. oryzae*, *P. chrysogenum*, *A. nidulans* and *A. terreus* required the presence of ATP and Mg^{2+} ions. A representative experiment is shown in Table 1. In these crude suspensions there was considerable synthesis of choline sulphate in the absence of added choline owing to the endogenous free and combined choline in the mycelia. Addition of 0·01 M-choline chloride and 12·5 mM-cysteine increased the amount of choline sulphate produced whereas 0·08 M-EDTA totally inhibited the synthesis. The enzyme system required for the choline sulphate synthesis was readily extracted from the acetone-dried mycelia by homogenizing with water. The crude suspensions and aqueous extracts

catalysed the transfer of $^{35}SO_4^{2-}$ ions from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate to choline chloride. The transfer was enzymic since boiled preparations were inactive in this respect. The specificity of the transferase, choline sulphokinase, has been examined and will be reported separately. Transfer of $^{35}SO_4^{2-}$ ions to choline from adenosine 5′[^{35}S]-sulphatophosphate in the absence of added ATP could not be demonstrated.

The acetone-dried mycelia of the fungi which accumulate choline sulphate in their mycelia during growth (Harada & Spencer, 1960) were all able to synthesize choline sulphate *in vitro* in the obligatory presence of added ATP and Mg^{2+} ions. All these fungi possessed choline sulphokinase and the adenosine 3′-phosphate 5′-phosphatosulphate-synthesizing activity (Table 2). The fungi without choline sulphate (Harada & Spencer, 1960) all produced adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate but did not transfer the $^{35}SO_4^{2-}$ ions of the compound to choline (Table 2). This was ascribed to a lack of choline sulphokinase rather than to the presence of an inhibitor of the transferase, since extracts of baker's yeast, *R. stolonifer* and *E. ashbyii* did not inhibit the synthesis of choline [^{35}S]sulphate from adenosine 3′-phosphate 5′[^{35}S]-sulphatophosphate and choline chloride by the choline sulphokinase of *A. oryzae*.

Competitive metabolism. Although the existence of the adenosine 3′-phosphate 5′-sulphatophosphate-synthesizing system in both the choline sulphate-producing fungi and the choline sulphate-less fungi tended to support the hypothesis that the two classes of fungi had a common method of sulphate utilization, i.e. through adenosine 3′-phosphate 5′-sulphatophosphate, the position of

Table 2. *Adenosine 3′-phosphate 5′-sulphatophosphate-synthesizing system and choline sulphokinase in fungi*

Aqueous suspensions of the fungi were tested by the methods described in the text.

	Choline sulphate formation		Synthesis of adenosine 3′-phosphate 5′-sulphato-phosphate *in vitro*	Choline sulphokinase *in vitro*
	*In vivo**	*In vitro*		
A. oryzae	+	+	+	+
A. nidulans	+	+	+	+
A. terreus	+	+	Not tested	+
A. sydowi	+	+	+	+
P. aurantio-brunneum	+	+	+	+
P. notatum	+	+	+	+
P. chrysogenum	+	+	+	+
P. citreo-roseum	+	+	+	+
R. stolonifer	−	−	+	−
E. ashbyii	−	−	+	−
N. gossypii	−	−	+	−
T. utilis	−	−	+	−
Baker's yeast	−	−	+	−

* Harada & Spencer (1960).

Table 3. *Competitive utilization of sodium sulphate for cystine synthesis by baker's yeast and* Aspergillus nidulans

The fungi were grown on media containing different sources of sulphur as indicated. After growth for 3 days (baker's yeast) or 5 days (*A. nidulans*) the mycelia were harvested, hydrolysed, the radioactive cystine was isolated after the addition of carrier cysteine and the specific activity measured. Details were as described in the text.

Fungus	Sulphur source	Radioactivity of cystine isolated from the mycelium (counts/sec./mg.)
Baker's yeast	$Na_2{}^{35}SO_4$	126
	$Na_2{}^{35}SO_4$ + choline sulphate	109
	[^{35}S]Choline sulphate	92
	[^{35}S]Choline sulphate + Na_2SO_4	1
A. nidulans	$Na_2{}^{35}SO_4$	138
	$Na_2{}^{35}SO_4$ + choline sulphate	120
	Choline [^{35}S]sulphate	121
	Choline [^{35}S]sulphate + Na_2SO_4	46

choline sulphate remained unclarified. Before further progress could be made it was essential to establish whether or not choline sulphate was an obligatory intermediate in sulphate utilization. Competitive-metabolism experiments were therefore carried out by growing fungi on media containing normal and radioactive sodium sulphate or choline sulphate, alone or in combinations, as the only sulphur sources. The rate of disappearance of ^{35}S from the media (Figs. 1, 2) and the specific activity of the cystine isolated from the mycelia were then measured (Table 3).

The six fungi chosen (three choline sulphate producers and three non-producers) individually appeared to utilize the sulphur of $Na_2{}^{35}SO_4$ or choline [^{35}S]sulphate, when present singly, to about the same degree, as shown by the rate of disappearance of ^{35}S from the media (Figs. 1, 2) and the weights of mycelia produced (Table 4). In the presence of non-radioactive choline sulphate the

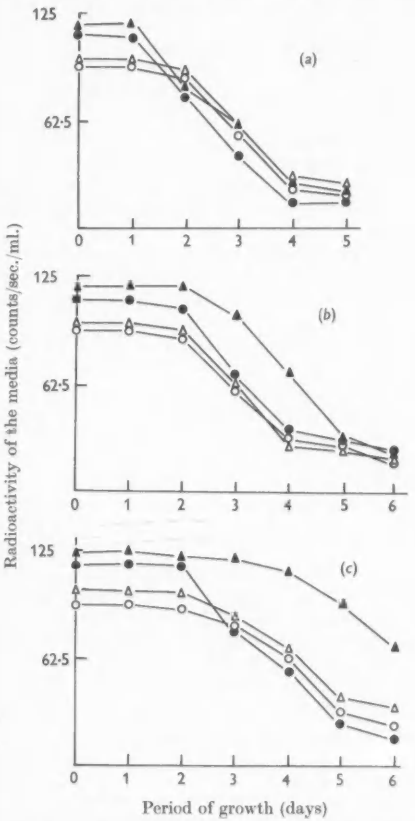


Fig. 1. Competitive utilization of Na_2SO_4 and choline sulphate by choline sulphate-producing fungi: (*a*) *A. nidulans*; (*b*) *P. chrysogenum*; (*c*) *P. aurantio-brunneum*. Fungi were grown on 200 ml. of the glucose–asparagine medium containing sulphur-containing supplements as indicated. ○, Medium containing $Na_2{}^{35}SO_4$; ●, choline [^{35}S]sulphate; △, $Na_2{}^{35}SO_4$ plus choline sulphate; ▲, Na_2SO_4 plus choline [^{35}S]sulphate. Na_2SO_4 was incorporated in all cases at a concentration of 34·5 mg. of $SO_4{}^{2-}$ ions/l. and the choline sulphate at a concentration equivalent to 25 mg. of $SO_4{}^{2-}$ ions/l.

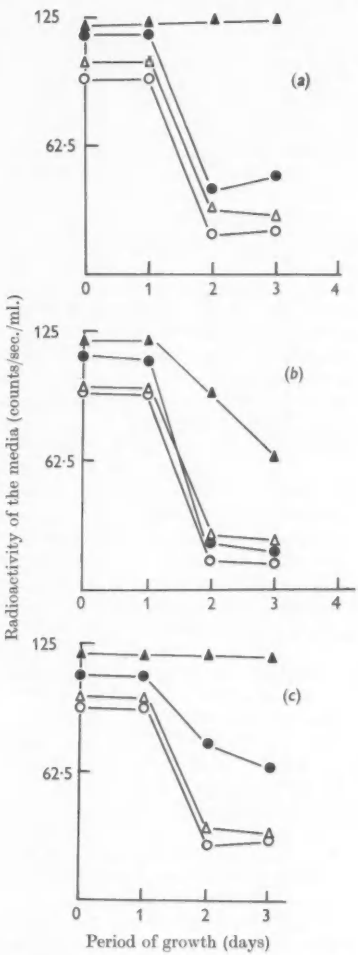


Fig. 2. Competitive utilization of Na_2SO_4 and choline sulphate by choline sulphate-less fungi: (*a*) baker's yeast; (*b*) *Torulopsis utilis*; (*c*) *Rhizopus stolonifer*. Details were as given in Fig. 1.

Table 4. *Dry weights of mycelia produced in the competitive-utilization experiment*

Fungi were grown in 200 ml. of glucose–asparagine medium with sulphur-containing supplements as indicated and harvested on the final day. Details were as described in the text and Figs. 1 and 2. Mycelial dry weights are recorded in milligrams.

Sulphur source	*A. nidulans*	*P. chrysogenum*	*P. aurantio-brunneum*	Baker's yeast	*T. utilis*	*R. stolonifer*
$Na_2{}^{35}SO_4$	467	561	304	666	484	407
$Na_2{}^{35}SO_4$ + choline sulphate	538	753	306	667	547	397
Choline [^{35}S]sulphate	544	685	411	683	511	321
Choline [^{35}S]sulphate + Na_2SO_4	512	746	350	612	521	405

uptake of $Na_2{}^{35}SO_4$ was the same as in the absence of the sulphate ester. The rate of uptake of ^{35}S from the media containing choline [^{35}S]sulphate plus Na_2SO_4 could not be distinguished from the rates of uptake from the other media with *A. nidulans* and *P. chrysogenum* but *P. aurantio-brunneum* and the choline sulphate-less *T. utilis* showed a much slower rate of uptake. Baker's yeast and *R. stolonifer* showed no uptake of ^{35}S from this medium.

These results suggested that Na_2SO_4 was used preferentially to choline sulphate as a source of sulphur by *P. aurantio-brunneum*, *T. utilis*, *R. stolonifer* and baker's yeast. *A. nidulans* and *P. chrysogenum* showed an anomalous behaviour in that they both appeared to take up choline sulphate and Na_2SO_4 at approximately the same rate from the medium containing both sulphur sources. It should be pointed out that in experiments of this type only uptake of ^{35}S into the mycelium, and not utilization of ^{35}S, was being measured. *A. nidulans* and *A. oryzae* are both known to store relatively large amounts of choline sulphate in their mycelia (Harada & Spencer, 1960), whereas *P. aurantio-brunneum* contains only small amounts of the ester. It is also significant that although the data in Fig. 1 would suggest that *A. nidulans* and *P. chrysogenum* took up almost all the choline sulphate and Na_2SO_4 when both sulphur sources were present together, there was very little difference between the weights of mycelia formed when the two sulphur sources were present either together (equivalent to 59·5 mg. of $SO_4{}^{2-}$ ions/l.) or singly (34·5 mg. of $SO_4{}^{2-}$ ions/l. as Na_2SO_4 or 25·0 mg. of $SO_4{}^{2-}$ ions/l. as choline sulphate) (Table 4). One interpretation of the results for *A. nidulans* and *P. chrysogenum* was that they showed only high rates of uptake and storage, rather than utilization, of choline sulphate and that although choline sulphate could be utilized as a sulphur source Na_2SO_4 was used preferentially.

Proof of this interpretation was provided by the specific radioactivities of the cystine isolated from the cells of baker's yeast and the mycelia of *A. nidulans* grown on the various media (Table 3). The radioactivity of the cystine from baker's yeast grown on choline [^{35}S]sulphate plus Na_2SO_4 was less than 1 % of that grown on either $Na_2{}^{35}SO_4$ alone or $Na_2{}^{35}SO_4$ plus non-radioactive choline sulphate. The comparable figure for *A. nidulans* was approximately 30 %.

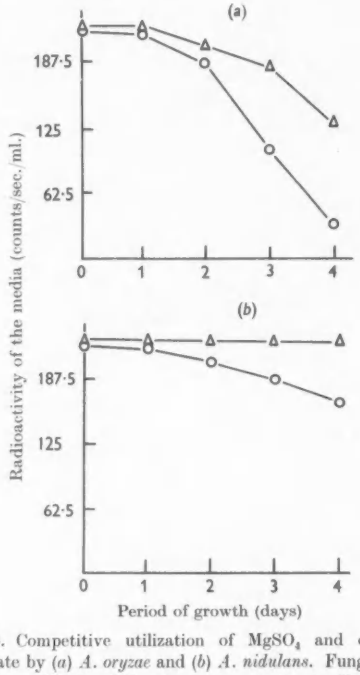


Fig. 3. Competitive utilization of $MgSO_4$ and choline sulphate by (*a*) *A. oryzae* and (*b*) *A. nidulans*. Fungi were grown on 200 ml. of the glucose–asparagine medium and harvested after 7 days' growth. ○, $MgSO_4$ (198 mg. of $SO_4{}^{2-}$ ions/l.) plus an insignificant amount of $Na_2{}^{35}SO_4$; △, $MgSO_4$ (198 mg. of $SO_4{}^{2-}$ ions/l.) plus choline sulphate (at a concentration equivalent to 198 mg. of $SO_4{}^{2-}$ ions/l.) and an insignificant amount of $Na_2{}^{35}SO_4$. The dry weights of mycelia were: *A. oryzae*, 675 mg. on $MgSO_4$ medium, 703 mg. on $MgSO_4$ + choline sulphate medium; *A. nidulans*, 650 mg. on $MgSO_4$ medium, 635 mg. on $MgSO_4$ + choline sulphate medium.

It was concluded that whereas baker's yeast can grow on either sulphur source independently it utilizes Na_2SO_4 to the exclusion of choline sulphate

when both sulphur sources are present; *A. nidulans* can use both sources when they are present simultaneously but Na_2SO_4 was the more readily assimilated. This suggests that the method of utilization of the sulphur of choline sulphate may be different in yeast and *A. nidulans*, but in neither case would it appear that choline sulphate was an obligatory intermediate in the utilization of inorganic sulphate.

With this knowledge it now became possible to explain the competitive-metabolism experiment of Itahashi (1954), in which the sulphate concentration was much higher than in the present series. A preliminary experiment which was carried out with 198 mg. of SO_4^{2-} ions/l. closely repeated the results of Itahashi (Fig. 3). The rate of uptake of ^{35}S by *A. oryzae* was slower from the $Na_2{}^{35}SO_4$ plus choline sulphate medium than from that containing $Na_2{}^{35}SO_4$ alone as the sulphur source. With *A. nidulans* the uptake of radioactivity from the medium containing $Na_2{}^{35}SO_4$ plus choline sulphate was barely detectable (Fig. 3). Both these fungi store choline sulphate and when $Na_2{}^{35}SO_4$ was used as the sole sulphur source a large amount of the $^{35}SO_4$ was presumably used for the formation and storage of choline sulphate. When $Na_2{}^{35}SO_4$ plus choline sulphate was present in the medium the choline sulphate was absorbed and stored directly and the $^{35}SO_4^{2-}$ ion was then only required for growth purposes. With these high sulphur concentrations the amount of $^{35}SO_4^{2-}$ ion used for growth was only a small percentage of the added amount and thus little uptake of $^{35}SO_4^{2-}$ ions was observed.

Utilization of sulphate esters as sulphur sources for fungal growth. *A. oryzae* grew well on media containing various aliphatic and aromatic sulphate esters as the sole sources of sulphur (Table 5). A certain amount of growth occurred in the absence of added sulphur, and this can probably be attributed to the presence of small sulphur reserves in the spores or to sulphur compounds introduced in the inoculum or as trace impurities in the media.

One way in which the sulphur of sulphate esters could have been made available to the fungi was by release of SO_4^{2-} ions through the action of a sulphatase or by degradation of the organic moiety of the compounds followed by the further metabolism of the liberated SO_4^{2-} ions. For example, *A. oryzae* contains an arylsulphatase and during growth on nitrocatechol sulphate the medium turned red, indicating that nitrocatechol had been released. The red subsequently disappeared as the nitrocatechol was metabolized. It would be expected that the utilization of the sulphur of sulphate esters in this way would be strongly inhibited by the incorporation of $BaCl_2$ into the media to precipitate the liberated SO_4^{2-} ions. This experiment was carried out (Table 5) and, whereas the growth of *A. oryzae* on choline sulphate was unaffected by barium, growth on the other sulphate esters, apart

Table 5. *Utilization of sulphate esters as sulphur sources for* Aspergillus oryzae *in the presence and absence of barium chloride*

A. oryzae was grown in 200 ml. of Czapek-Dox medium in which $MgSO_4$ was replaced by an equivalent amount of $MgCl_2$. Sulphur sources were incorporated at a concentration of 192 mg. of SO_4^{2-} ion/l. and $BaCl_2$ (1·0 g./l.) was added to one series of media. The mycelial dry weights are quoted as percentages of the dry weight of the mycelium when cysteine was the sulphur source.

Sulphur source	Dry wt. of mycelium: $BaCl_2$ absent	Dry wt. of mycelium: $BaCl_2$ present
None	10	5
Choline sulphate	101	105
Na_2SO_4	113	11
Sinigrin	80	13
Cortisone 21-sulphate	78	20
Phenyl sulphate	60	21
Nitrocatechol sulphate	101	25
Ethyl sulphate	83	30
Chondroitin sulphate A	51	69
2:4-Dichlorophenoxyethyl sulphate	87	78
Cysteine hydrochloride	100	100
Weight of mycelium with cysteine hydrochloride as sulphur source (mg.)	1·46	1·05

Table 6. *Utilization of different sulphur sources by fungi in the presence and the absence of barium chloride*

Fungi were grown in 200 ml. of Czapek-Dox medium in which $MgSO_4$ was replaced by an equivalent amount of $MgCl_2$. Sulphur sources were incorporated at a concentration of 192 mg. of SO_4^{2-} ions/l. and $BaCl_2$ (1·0 g./l.) was added to one series of media. The mycelial dry weights are given as percentages of the dry weight of the mycelium when cysteine was the sulphur source.

	Dry wt. of mycelium							
	Sulphur sources, $BaCl_2$ absent				Sulphur sources, $BaCl_2$ present			
	None	Na_2SO_4	Choline sulphate	Nitrocatechol sulphate	None	Na_2SO_4	Choline sulphate	Nitrocatechol sulphate
R. stolonifer	16	121	89	126	10	22	62	81
Baker's yeast	11	100	100	79	13	97	102	77
T. utilis	7	77	83	100	6	20	103	100
E. ashbyii	20	176	121	83	20	19	133	87

from chondroitin sulphate and 2:4-dichlorophenoxyethyl sulphate, was strongly inhibited.

The experiment was repeated in part with several choline sulphate-less fungi with cysteine, SO_4^{2-} ions, nitrocatechol sulphate or choline sulphate as the sole sulphur source (Table 6). Both sulphate esters were good sources of sulphur for all the fungi in the presence as well as in the absence of barium. With baker's yeast, the good growth on Na_2SO_4 in the presence of excess of $BaCl_2$ can be explained as the utilization of the very small concentration of $BaSO_4$ which remains in solution (2·3 mg./l. at 18°). The similar good growth on the sulphate esters would therefore have been expected. The high growth rate of *R. stolonifer*, *T. utilis* and *E. ashbyii* on the sulphate esters in the presence of $BaCl_2$ was unexpected since there was very little growth on Na_2SO_4 in the presence of $BaCl_2$. Two explanations may be advanced: (1) that the sulphur of choline sulphate and nitrocatechol is converted into a reduced form of organically combined sulphur without appearing as SO_4^{2-} ions; (2) that SO_4^{2-} ions are produced from the two sulphate esters but are further metabolized so rapidly at low concentration that $BaSO_4$ does not form. This could also happen if the mycelial membranes are not permeable to Ba^{2+} ions.

Sulphur storage in fungi. Much of the evidence indicated that choline sulphate could act as a store of sulphur. To test this suggestion fungi were grown for 4 days in shaking culture in 200 ml. of the glucose–asparagine medium. The mycelia of each species was harvested on sterilized muslin and the excess of water was removed from the collected mycelia by pressing between sterilized filter papers. The semi-dry mycelial mass was weighed and divided into three approximately equal portions, each of which was then weighed. One portion was used as the inoculum for 200 ml. of the glucose–asparagine medium, another portion was used as the inoculum for 200 ml. of the same medium in which the $MgSO_4$ had been replaced by $MgCl_2$ and the remaining portion was dried *in vacuo* over P_2O_5 for the calculation of the dry weights of the mycelia used as the inocula. After 24 hr. further growth in the new media the mycelia were reharvested, dried and weighed. With *P. chrysogenum* and *R. stolonifer* a division of the collected mycelia into five portions was made so as to allow collection after 13 and 24 hr. further growth in the new media. Although absolute asepsis was impracticable during the transfer operations no contamination during the second period of growth was observed.

The results (Table 7) showed that the choline sulphate-producing fungi *A. oryzae* and *P. chrysogenum* grew well on sulphur-deficient media. After 13 hr. further growth there was no difference in the weights of new mycelia between that grown on the sulphate-containing medium and that grown on the sulphate-deficient medium. After 24 hr. the growth on the sulphate-containing medium exceeded that on the sulphate-deficient medium, indicating that the sulphur reserves of the mycelial inoculum in the latter case had been used. However, these reserves were sufficient to support a 150% increase in growth of *A. oryzae* and 74% increase of *P. chrysogenum*. On the other hand, very little further growth of the choline sulphate-less fungi occurred on the sulphate-deficient medium, indicating that these fungi did not contain any substantial sulphur reserves.

Chromatography of extracts of *A. oryzae* and *P. chrysogenum* (cf. Harada & Spencer, 1960) showed that when these fungi were grown in Czapek-Dox medium containing $^{35}SO_4^{2-}$ ions they stored ^{35}S both as $^{35}SO_4^{2-}$ ions and as choline [^{35}S]sulphate. The latter compound predominated in the ratio of approximately 10:1 when all nutrients in the medium were present in excess. On transfer of the fungi to media deficient in sulphate and when the fungi were grown to exhaustion, chromatography

Table 7. *Sulphur storage by fungi*

Fungi were grown in 200 ml. of the glucose–asparagine medium for 4 days. Portions of the mycelia were then transferred to fresh media, which was either complete or totally deficient in sulphate, and the increase in weight of the mycelium after 13 hr. or 24 hr. further growth was measured.

		Increase in dry wt. of mycelium (%)	
Choline sulphate-producing fungi	Sulphate in medium	13 hr.	24 hr.
A. oryzae	−	.	151
	+	.	277
P. chrysogenum	−	62	73
	+	67	182
Choline sulphate-less fungi			
R. stolonifer	−	.	14
	+	.	144
M. hiemalis	−	.	5
	+	.	44

of mycelial extracts showed that the mycelial store of both $^{35}SO_4^{2-}$ ions and choline [^{35}S]sulphate had been completely used.

DISCUSSION

Kaji & McElroy (1958) had previously shown that extracts of *P. sydowi* formed choline sulphate in the obligatory presence of ATP and SO_4^{2-} ions. From this and other results they suggested that adenosine 3′-phosphate 5′-sulphatophosphate was formed and that this acted as a donor of sulphate for the synthesis of choline sulphate under the influence of a transferase, choline sulphokinase. This suggestion has now been confirmed by the demonstration that a number of fungi can produce adenosine 3′-phosphate 5′-sulphatophosphate and that they can transfer sulphate from the exogenous compound to choline. No transfer of sulphate from adenosine 5′-sulphatophosphate was observed. Kaji & Gregory (1959) have also recently confirmed that transfer of sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to choline takes place in extracts of *P. sydowi*.

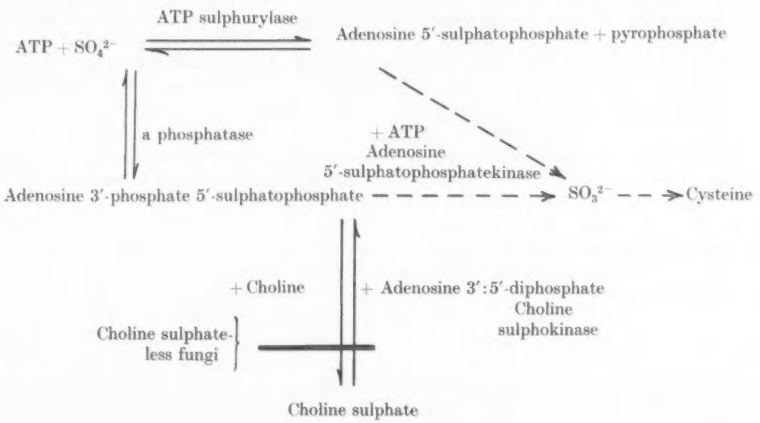
All the fungi tested (Table 2) produced adenosine 3′-phosphate 5′-sulphatophosphate but the fungi which did not accumulate choline sulphate *in vivo* (Harada & Spencer, 1960) lacked the enzyme choline sulphokinase. It would seem a reasonable generalization to suggest that all fungi which can utilize inorganic sulphate as a source of sulphur for growth can produce adenosine 3′-phosphate 5′-sulphatophosphate. The distribution of choline sulphate synthesis in fungi can therefore be discussed in terms of the presence or absence of choline sulphokinase (cf. Harada & Spencer, 1960; Ballio *et al.* 1960).

Work with mutant strains of *A. nidulans* (Hockenhull, 1949; Shepherd, 1956) has shown that the reduction of sulphate to sulphite is an obligatory stage in the formation of cysteine from inorganic sulphate. The reaction

$$SO_4^{2-} + 2H \rightarrow SO_3^{2-} + H_2O$$

is an endergonic reaction with a standard free energy change of +14 kcal. (Postgate, 1956), and it is therefore not surprising to learn that reduction of sulphate *in vitro* by yeast requires ATP, Mg^{2+} ions and reduced triphosphopyridine nucleotide (TPNH) (Wilson & Bandurski, 1958; Hilz & Kittler, 1958). The ATP and Mg^{2+} ions can be replaced by adenosine 3′-phosphate 5′-sulphatophosphate (Wilson & Bandurski, 1958) or by *p*-nitrophenyl sulphate plus adenosine 3′:5′-diphosphate in the presence of phenolsulphokinase (Hilz & Kittler, 1958), and it is further known that adenosine 3′-phosphate 5′-sulphatophosphate can be reduced by TPNH (Wilson & Bandurski, 1958). The evidence strongly suggests that adenosine 3′-phosphate 5′-sulphatophosphate must be formed before sulphate can be reduced although it does not rule out the possibility that the immediate precursor of SO_3^{2-} ions is adenosine 5′-sulphatophosphate. Yeast and other fungi are known to contain enzymes which degrade adenosine 3′-phosphate 5′-sulphatophosphate to adenosine 5′-sulphatophosphate (B. Spencer, unpublished work), and recently Peck (1959) and Ishimoto (1959) have shown that adenosine 5′-sulphatophosphate and not the former compound is the substrate which is reduced by extracts of *Desulphovibrio desulphuricans*. Whether the same reducing system exists in yeast, where its purpose is sulphate assimilation, and in *Desulphovibrio*, where sulphate serves as the terminal electron acceptor, is not known.

The following tentative scheme for sulphate utilization in fungi can be proposed.



In this scheme choline sulphate is envisaged as a storage compound containing sulphate in a high-energy state. On demand the sulphate can be transferred to adenosine 3′:5′-diphosphate to give adenosine 3′-phosphate 5′-sulphatophosphate which can form sulphite. If, in the reduction, adenosine 3′:5′-diphosphate is regenerated, as envisaged by Hilz & Kittler (1958), then only a catalytic amount of adenosine 3′:5′-diphosphate would be required for the utilization of large amounts of choline sulphate.

Some evidence has been obtained to support the position of choline sulphate in this scheme. It has long been known (Egami & Itahashi, 1951) that choline sulphate can be utilized as a sulphur source without the appearance of inorganic sulphate, and this was confirmed in the present investigation when it was shown that in the presence of Ba^{2+} ions choline sulphate remains a good source of sulphur for growth but that several other sulphate esters do not. Another fact supporting the view that sulphate is transferred from choline sulphate to an acceptor rather than sulphate being liberated by degradation of the carbon chain of the choline is that choline sulphate can act as a source of choline for the choline-less mutant of *N. crassa* (Stevens & Vohra, 1955; Harada & Spencer, 1960). Furthermore, the choline-less mutant of *N. crassa* and the fungi listed in Table 2 did not possess any sulphatase activity *in vitro* towards choline sulphate when tested by a sensitive radioactive method (B. Spencer, unpublished work).

The results of the competitive-metabolism experiment are also consistent with the proposed scheme. In the choline sulphate-less fungi, choline sulphate did not affect the conversion of inorganic sulphate into cysteine and this is simply explained from the scheme as being due to the lack of choline sulphokinase. In the choline sulphate-producing fungi the finding that inorganic sulphate and choline sulphate can act as alternative and competing sources of sulphur for cysteine production is compatible with the proposed scheme. The fungi which accumulate choline sulphate in their mycelia during growth on a medium containing excess of sulphate could utilize the stored sulphur of the sulphate ester for further growth when transferred to a medium lacking any sulphur source. The choline sulphate-less fungi showed little further growth under these circumstances. These facts are again readily explained by the scheme. An important confirmation of the proposed method of utilization of choline sulphate would be the demonstration of the formation *in vitro* of adenosine 3′-phosphate 5′-sulphatophosphate from choline sulphate and adenosine 3′:5′-diphosphate under the influence of choline sulphokinase. No evidence of this has yet been obtained (Kaji & Gregory, 1959). However, similar reactions are known to occur with other sulphate esters (Lipmann, 1958).

If the proposed scheme should prove correct choline sulphate will assume a somewhat unique role in fungi as a store of easily assimilated sulphate existing in an activated state. The position would be somewhat akin to the role of guanidinium phosphates in animals, although the parallel should not be drawn too closely. In conditions of adequate sulphur nutrition choline sulphate would accumulate in the mycelia and then be re-utilized when sulphur supplies became subminimal. This property could be considered to have been of evolutionary advantage since it is limited to the higher orders of fungi (Harada & Spencer, 1960; Ballio *et al.* 1960). Some confirmation of the idea of choline sulphate as a storage compound has come from the work of Takebe & Yanagita (1959), who found an unidentified sulphur-containing compound in the conidiospores of *Aspergillus niger* which was utilized for the formation of sulphur-containing amino acids during germination. This compound has now been shown to be choline sulphate (I. Takebe, personal communication) and it occurred to the extent of 1·4% of the fresh spore weight.

SUMMARY

1. Choline sulphate synthesis *in vitro* by extracts of a number of fungi in the presence of adenosine triphosphate and Mg^{2+} ions was shown to involve the intermediate formation of adenosine 3′-phosphate 5′-sulphatophosphate followed by the enzymic transfer of sulphate from this compound to choline. Adenosine 3′-sulphatophosphate could not act as a sulphate donor.

2. All fungi tested produced adenosine 3′-phosphate 5′-sulphatophosphate *in vitro* but those fungi which did not accumulate choline sulphate in their mycelia *in vivo* could not transfer sulphate from adenosine 3′-phosphate 5′-sulphatophosphate to choline. The distribution of choline sulphate production can therefore be discussed in terms of the presence or absence of the transferase, choline sulphokinase.

3. In competitive-metabolism experiments choline sulphate did not affect the utilization of SO_4^{2-} ions by choline sulphate-less fungi without choline sulphate, including baker's yeast. In the choline sulphate-producing fungi choline sulphate and SO_4^{2-} ions were competing sources of sulphur for cysteine synthesis.

4. Choline sulphate served as a good source of sulphur for the growth of choline sulphate-producing fungi both in the absence and the presence of excess of Ba^{2+} ions. Other sulphate esters, apart from chondroitin sulphate and 2:4-dichlorophenoxyethyl sulphate, were poor sources of

sulphur in the presence of Ba^{2+} ions. Choline sulphate and nitrocatechol sulphate were good sulphur sources for the growth of choline sulphate-less fungi both in the absence and the presence of Ba^{2+} ions.

5. When choline sulphate-producing fungi were grown on an adequate sulphur medium a store of choline sulphate accumulated in the mycelia which could be used to maintain further growth when the fungi were transferred to a medium totally deficient in sulphur of any form. In the same circumstances no further growth of choline sulphate-less fungi occurred on the new sulphur-deficient medium.

6. Choline sulphate in fungal mycelia is envisaged as a store of easily assimilated sulphate existing in an activated state. The pathway of utilization is thought to involve transfer of sulphate from choline sulphate to adenosine 3′:5′-diphosphate with the formation of adenosine 3′-phosphate 5′-sulphatophosphate and the subsequent reduction of adenosine 3′-phosphate 5′-sulphatophosphate to give SO_3^{2-} ions and eventually cysteine.

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Condensed Tannins

6. BIOCHEMICAL AND STEREOCHEMICAL INTERRELATIONSHIPS OF (+)-FUSTIN FROM BLACK-WATTLE HEARTWOOD (*ACACIA MOLLISSIMA*)*

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Condensed tannins from the heartwood of *Acacia mollissima* consist of a molecular gradation (290–1650) of polyphenolic compounds (Roux & Evelyn, 1960) in which (+)-7:3′:4′-trihydroxyflavan-3:4-diol [(+)-mollisacacidin, Keppler (1957)] is the predominant single component. Polymeric leuco-fisetinidin tannins form the major fraction of the heartwood polyphenols and appear to originate from this monomeric 'leuco-fisetinidin' flavan-3:4-diol (Roux, 1958). Other monomeric flavonoid substances of similar structural pattern are also present. The provisional identification of fustin and fisetin (Roux & Evelyn, 1960) is confirmed in this study, and their probable mechanism of biogenesis

* Part 5: Evelyn, Maihs & Roux (1960).

is discussed. The stereochemical interrelationships of various fustins, and the synthesis of (+)-mollisacacidin are also described.

EXPERIMENTAL AND RESULTS

All melting points are uncorrected. Mixed melting points were carried out as before (Roux & Maihs, 1960). Analyses of C, H, methoxyl groups and acetyl groups were by Weiler and Strauss, Oxford, and by Pascher, Bonn. Infrared comparisons were made by Dr J. R. Nunn, National Chemical Research Laboratory, C.S.I.R., Pretoria. A 2 dm. tube was used for optical rotations.

Selection of wattle heartwood. Radial examination by two-dimensional paper chromatography (Roux, 1958) of heartwoods of wattle trees, felled in plantations after the normal 8- to 10-year rotation (average wood diameter 7 in.), showed the predominant presence of (+)-mollisacacidin, but relatively low concentrations of fustin and fisetin. In older specimens, estimated to be at least 50 years old (average wood diameter 20 in.) the concentration of (+)-mollisacacidin was similarly high in the peripheral heartwood and persisted with progressively declining concentration into the central heartwood. Both fustin and fisetin, on the other hand, were in low concentration in the peripheral heartwood but increased slightly towards the central heartwood (cf. Table 2). These were observed in each of four 50-year-old specimens of *A. mollissima* examined. The heartwoods of the older trees were selected for the isolation of fustin and fisetin.

Extraction of heartwood and separation of components. The damp heartwood of the freshly cut tree was drilled and the damp drillings (2·6 kg.) were extracted with ethyl acetate (7·5 l.) in two extractions of 48 hr. each. The extracts were dried in a rotary evaporator under vacuum and the powdered residue (37 g.) was extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus for 6 hr. The wax-free residue (30 g.) was dissolved in 400 ml. of the lower phase of a water–butan-2-ol–'benzine' (5:3:2) mixture and introduced into the first eight tubes of a 160-tube, 50 ml. underphase, fully automatic Craig countercurrent machine (Glasapparatebau Göttingen-Helmut Rettberg). The upper and lower phases of the above mixture were used for countercurrent separation, and after 160 transfers the contents of tubes 120–145 and 146–160 were run off. The organic phase was separated and the aqueous phase extracted with ethyl acetate in each instance. The combined organic phase and ethyl acetate extracts of tubes 120–145 were decreased to 40 ml. as before, and streaked on to eight sheets (5 ml./sheet) of 22½ in. × 18½ in. Whatman no. 3 chromatographic paper. The chromatograms were developed by upward migration in 2% acetic acid. The fustin band (R_F 0·43) was located with reference to the (+)-mollisacacidin band [toluene-*p*-sulphonic acid, Roux (1957*b*)] on a test strip. The fustin band, which migrated immediately behind this leuco-fisetinidin band (R_F 0·57), was cut and stripped in 70% ethanol. Fustin (250–300 mg.) crystallized from a concentrate of the eluents. Fisetin was present, amongst other components, in tubes 146–160 of the Craig machine.

(+)-*Fustin.* Fustin from the Craig-countercurrent and paper-chromatographic separations, was recrystallized from water as fine needles, m.p. 209–211°. The m.p. in soda glass was variable and apparently dependent on the rate of heating. In Pyrex glass (cf. Hillis, 1952; Laidlaw & Smith, 1958) the m.p. was elevated to 228–229° and consistent results were obtained. Fustin, dried over $CaCl_2$, was analysed as a semi-hydrate (Found: C, 60·2; H, 4·3; loss at 110°, 3·2. $C_{15}H_{12}O_6$,0·5H_2O requires C, 60·6; H, 4·4; H_2O, 3·0%). After 2 hr. at 110° the fustin became anhydrous [Found: C, 62·4; H, 4·3. $C_{15}H_{12}O_6$ requires C, 62·5; H, 4·2%. $[\alpha]_D^{23}$ +28·3±1·1° in acetone–water 1:1 (*c*, 0·9)]. (+)-Fustin and (−)-fustin showed identical infrared-absorption curves over the range 2·5–15 μ, and these were almost identical with the infrared curve of (±)-fustin over the same range.

Tetra-acetyl-(+)-fustin. (+)-Fustin (500 mg.) was acetylated with 2 ml. of pyridine and 3 ml. of acetic anhydride at room temperature for 5 hr. The product was poured into water and, after hardening overnight, was recrystallized twice from ethanol (0·3 g.), m.p. 116–119°. $[\alpha]_D^{25}$ +24·4± 0·8° in tetrachloroethane (*c*, 1·3) (Found: C, 60·2; H, 4·50; CO·CH_3, 38·1. $C_{23}H_{20}O_{10}$ requires C, 60·5; H, 4·4; CO·CH_3, 37·7%). Tetra-acetyl derivatives of (+)-, (−)- and (±)-fustin showed identical infrared-absorption curves over the range 2·5–15 μ.

O-Trimethyl-(+)-fustin. (+)-Fustin (500 mg.) in 50 ml. of methanol was methylated with excess of diazomethane at −5° as described for 7:3′:4′-trimethoxyflavan-3:4-diol (Roux & Evelyn, 1958). The product (0·3 g.) was recrystallized twice from ethanol, m.p. 138–140°, $[\alpha]_D^{23}$ −44·3±1·6° in tetrachloroethane (*c*, 1·3) (Found: C, 65·5; H, 5·5; OMe, 27·6. $C_{18}H_{21}O_9$ requires C, 65·5; H, 5·6; OMe, 28·2%). The infrared-absorption curves of *O*-trimethyl-(+)-fustin and -(±)-fustin were identical over the range 2·5–15 μ.

Acetyl-O-trimethyl-(+)-fustin. *O*-Trimethyl-(+)-fustin (90 mg.) was acetylated with 0·4 ml. of pyridine and 0·4 ml. of acetic anhydride for 3 hr. at room temperature. The product was poured into water and recrystallized from ethanol (60 mg.), m.p. 119–121°, $[\alpha]_D^{25}$ +2·1±0·3° in tetrachloroethane (*c*, 0·8) (Found: C, 64·6; H, 5·6; CO·CH_3, 11·1; OMe, 26·9. $C_{20}H_{20}O_7$ requires C, 64·5; H, 5·4; CO·CH_3, 11·6; OMe, 25·0%).

(±)-Fustin and derivatives. (±)-Fustin from *Rhus glabra* was isolated as described by Roux & Freudenberg (1958) and the derivatives were prepared as described above. This racemic mixture showed low negative rotation $[\alpha]_D^{21}$ −2·4°. (Roux & Freudenberg, 1958) and its m.p. 214–216° in soda glass was raised to 226–228° in Pyrex glass. Tetra-acetyl-(±)-fustin, m.p. 145° (Found: C, 60·4; H, 4·3; CO·CH_3, 36·8. Calc. for $C_{23}H_{20}O_{10}$: C, 60·5; H, 4·4; CO·CH_3, 37·7%). *O*-Trimethyl-(±)-fustin, m.p. 140–143°. Acetyl-*O*-trimethyl-(±)-fustin, m.p. 144–145°.

Tetra-acetyl-(−)-fustin. (−)-Fustin (50 mg.) (Freudenberg & Weinges, 1959; Weinges, 1959) from *Rhus cotinus*, kindly supplied by Professor K. Freudenberg and Dr K. Weinges, was acetylated with 0·2 ml. of pyridine and 0·3 ml. of acetic anhydride at room temperature for 3 hr. The product was poured into 3 ml. of water, allowed to harden overnight and crystallized from ethanol (46 mg.), m.p. 115–116° [m.p. 117–118° found by Weinges (1959)], $[\alpha]_D^{23}$ −25·2±1·1° in tetrachloroethane (*c*, 0·8). The infrared-absorption curves of tetra-acetyl-(−)-fustin and tetra-acetyl-(+)-fustin were superimposable over the range 2·5–15 μ.

Comparison of melting points of (+)-fustin, (−)-fustin, (±)-fustin and their tetra-acetyl derivatives. In Pyrex glass

the m.p.'s of the above-listed fustins were all elevated to the range 226–230°. An equimolecular mixture of (+)- and (−)-fustin showed no elevation of m.p. A mixed m.p. of a molecular mixture of tetra-acetyl-(+)-fustin (m.p. 116–119°) and tetra-acetyl-(−)-fustin (m.p. 115–116°) showed an elevation to 150–151·5°. The latter agrees approximately with the m.p. (145° and 147–148°) of tetra-acetyl-(±)-fustin from *R. glabra*, and exactly with the m.p. of tetra-acetylfustin (150–151°) from *Rhus succedanea* (Oyamada, 1934, 1939). A mixed m.p. of the artificial racemic mixture and that derived from the natural racemate from *R. glabra* showed no depression (m.p. 147–149°). A comparison of the melting points and optical rotations of the various fustins is shown in Table 1.

Paper chromatography of (+)-, (−)- *and* (±)-*fustin.* The naturally occurring fustins of *A. mollissima*, *R. cotinus* and *R. glabra* all have the same R_F values in partitioning mixtures [0·81 in water-saturated butan-2-ol, and 0·80 in butan-1-ol–acetic acid–water (4:1:5)], but show slight but reproducible differences in 2% acetic acid or water when run at identical concentrations on the same sheet of paper. In 2% acetic acid with reference substances (−)-robinetinidol (0·42) and (+)-catechin (0·36) the following R_F values were recorded: (−)-fustin, 0·35; (±)-fustin, 0·36; (+)-fustin, 0·37. As expected, with such small differences in R_F between (+)- and (−)-fustin, the racemic mixture showed no tendency to separate into its optical antipodes.

Hydrogenation of (+)-*fustin to* (+)-*mollisacacidin.* (+)-Fustin (500 mg.) from *A. mollissima* was hydrogenated in methanol with platinum oxide as catalyst (Freudenberg & Roux, 1954), as described in detail by Roux & Freudenberg (1958) for (±)-fustin. The theoretical volume of hydrogen was absorbed in 12 hr. The product after repeated recrystallization from water (69 mg.) melted at 125–129°, and showed no depression in a mixed m.p. (125–129°) with (+)-mollisacacidin from wattle heartwood; $[\alpha]_D^{23}$ +36·7°±1·3° in acetone–water (1:1) (*c*, 1·1). The product showed the same R_F as (+)-mollisacacidin in 2% acetic acid (0·55), but a distinctly higher R_F than that (0·49) of the enantiomorphous (−)-7:3′:4′-trihydroxyflavan-3:4-diol from *Schinopsis quebracho-colorado* (Clark-Lewis & Roux, 1958, 1959). The hydrogenation product showed the same R_F (0·76) as (+)- and (−)-7:3′:4′-trihydroxyflavan-3:4-diol in water-saturated butan-2-ol (Roux & Evelyn, 1958), and was readily converted into fisetinidin chloride (Roux, 1957*a*) on treatment with hot hydrochloric acid in propan-2-ol under pressure at 100°.

Isolation and identification of fisetin. The upper phases of tubes 146–160 from the Craig separation were concentrated to dryness as before. The concentrates (1·1 g.) were streaked on to Whatman no. 3 sheets and the chromatograms developed in 2% acetic acid as for (+)-fustin. The bands on the starting line fluoresced a brilliant greenish yellow under ultraviolet light and were eluted as before. The eluents were concentrated to dryness and the solid residue boiled with 500 ml. of N-sulphuric acid for 1 hr. The solution was filtered hot and extracted with ether after cooling. The yellow product obtained from evaporation of the ether was recrystallized twice from ethanol–water, yielding 3 mg. of yellow needles. The ultraviolet and visible range spectra (Found: $\lambda_{max.}$, 252, 320, 360 mμ and log $E^{1\%}_{1\,cm.}$ 2·62, 2·51, 2·73 respectively) were similar to that of fisetin derived from the refluxing of (±)-fustin in N-sulphuric acid in a current of air according to the method of Pew (1948) (Found: $\lambda_{max.}$ 250, 320, 365 mμ and log $E^{1\%}_{1\,cm.}$ 2·69, 2·59, 2·84 respectively) (cf. Kirby & White, 1955; Mitsuno & Yoshizaki, 1957). The infrared-absorption curves of the substance and of fisetin were identical over the range 2·5–15 μ.

Table 1. *Melting points and optical rotations of enantiomorphous fustins and their racemates*

Chadenson, Molho-Lacroix, Molho & Mentzer (1955) found m.p. 248° for fustin from *Gleditsia* (*Gleditschia*) *triacanthos*. Hasegawa & Shirato (1951) found m.p. 218° for fustin, 158° for tetra-acetylfustin and 143° for *O*-trimethylfustin from *Rhus vernicifera*. Values in parentheses are from the authors listed in the footnotes.

Compound	*A. mollissima* (+)-Fustin	*R. succedanea*	*R. glabra* (±)-Fustin	Racemic mixture of enantiomorphs	*Cotinus coggygria* (*R. cotinus*) (−)-Fustin
Phenol					
M.p. (Pyrex glass)	228–229°	—	226–228°	226–228°	228 230°
M.p. (soda glass)	209–211°	(216–218°)*	222° (213–215°)† (223–225°)§	—	(216–218°)§
$[\alpha]_D$	+28·3°	—	(−2·4°)‡	—	(−26°)§
Tetra-acetyl					
M.p.	116–119°	(150–151°)*	145° (147–148°)§	150–151·5°	115–116° (117–118°)§
$[\alpha]_D$	+24·4°	—	—	—	−25·5°
O-Trimethyl					
M.p.	138–140°	(143–144°)*	140–143° (141–142°)†	—	—
$[\alpha]_D$	−44·3°	—	—	—	—
Monoacetyl-*O*-trimethyl					
M.p.	119–121°	(142–143°)*	144–145°	—	—
$[\alpha]_D$	+2·1°	—	—	—	—

* Oyamada (1939). † Keppler (1957). ‡ Roux & Freudenberg (1958).
§ Freudenberg & Weinges (1959); Weinges (1959).

Radial distribution of (+)-fustin and (+)-mollisacacidin in black-wattle heartwoods. The cross-section of a freshly felled 50-year-old specimen of *A. mollissima*, of wood diameter 20 in., was sampled radially by drilling at the centre, 4 in. from the centre and 8 in. from the centre (heartwood periphery). Sapwood surrounding the heartwood had an average width of 2 in. The drillings (30 g.) from each position were extracted with methanol (150 ml.) with four extractions of 24 hr. each at room temperature. Residual polyphenols, which could be removed similarly by three successive extractions with acetone–water (1:1), consisted mainly of highly condensed tannins as shown by paper chromatography. Extraction of these tannins was not complete even at this stage. The solid extractives were redissolved in methanol or acetone–water (5% soln.), and spotted on two-dimensional paper chromatographic sheets in 0·5 and 1 mg. concentrations. (+)-Fustin and (+)-7:3′:4′-trihydroxyflavan-3:4-diol were dissolved in methanol (0·1% soln.) and applied to two-way paper chromatograms in 10 μg. increments over the range 10–90 μg. The densitometric method of Roux & Maihs (1960) with ammoniacal silver nitrate as spraying reagent was used for the estimation of (+)-fustin and (+)-mollisacacidin in each of the radial samples, and in the ethyl acetate extract of the whole bark (Table 2). The values for (+)-fustin and (+)-mollisacacidin in the 'combined methanol and acetone–water extracts' are lower than anticipated from the values of the 'methanol extract', when also comparing the percentages of extractives in Table 2. A possible explanation for this is that acetone–water extraction removes mainly polymeric condensed tannins, as indicated on paper chromatograms. These tannins are therefore present in much higher concentration at or near the origin on paper chromatograms of the combined extract, and may possibly occlude or retain a proportion of the flavonoid compounds resulting in the relatively low values obtained on estimation.

DISCUSSION

Fustin (2:3-dihydrofisetin), first member of the now relatively large group of 2:3-dihydroflavonols to be found in Nature, was isolated by Oyamada (1934, 1939) from *Rhus succedanea*. Oyamada failed to record the optical rotation of his fustin, and this omission was repeated in more recent work on fustins by others (Hasegawa & Shirato, 1951; Chadenson, Molho-Lacroix, Molho & Mentzer, 1955; Mitsuno & Yoshizaki, 1957; Keppler, 1957). Freudenberg & Weinges (1959) and Weinges (1959) were the first to record the isolation of a (−)-fustin from *R. cotinus*, and the present isolation of a (+)-fustin of similar but opposite rotation from *A. mollissima* suggested that these substances were pure stereoisomers and possibly also enantiomorphs. This conjecture was proved by comparing the tetra-acetates of (+)- and (−)-fustin. The acetyl derivatives not only had equal and opposite rotations (Table 1) but similar and lower melting points (116–119°) compared with Oyamada's (1939) tetra-acetylfustin (150–151°) and tetra-acetyl-(±)-fustin from *R. glabra* (146–147°). A molecular mixture of tetra-acetyl-(+)-fustin and tetra-acetyl-(−)-fustin gave a melting-point elevation to 150–151·5° and proved the enantiomorphous nature of the parent compounds. *R. glabra* was shown to contain the natural racemate by a mixed melting point of tetra-acetyl-(±)-fustin and the artificial racemate. These interrelationships were confirmed by comparison of the infrared-absorption curves of the enantiomorphous fustins, the natural racemate and their derivatives. Further proof was also provided by the conversion of (+)-fustin into (+)-7:3′:4′-trihydroxyflavan-3:4-diol, which is enantiomorphous (Clark-Lewis & Roux, 1958, 1959) with the (−)-leucofisetinidin obtained by Freudenberg & Weinges (1959) by the hydrogenation of (−)-fustin.

From melting-point data in Table 1 it may be concluded that Oyamada's fustin was also a natural racemate similar to that in *Rhus glabra*, and similar racemates apparently also occur in *Rhus vernicifera* (Hasegawa & Shirato, 1951) and in

Table 2. *Estimation of (+)-fustin and (+)-mollisacacidin in radial samples of black-wattle heartwood from a 50-year-old tree*

Location of sample	Extract as % of damp-wood weight	(+)-Fustin (as % of solids in extract)*	(+)-Mollisacacidin (as % of solids in extract)*
Methanol extract			
Heartwood periphery	9·0	2·1	19·6
Middle heartwood	5·3	3·3	11·8
Central heartwood	5·1	2·6	5·9
Combined methanol and acetone–water extracts			
Heartwood periphery	11·6	1·3	13·8
Middle heartwood	8·0	2·3	7·0
Central heartwood	8·3	2·1	2·8
Ethyl acetate extract of whole heartwood	1·2	3·5	5·5

* The radial distribution of (+)-mollisacacidin and (+)-fustin represented is the pattern commonly encountered in the heartwood [compare radial distribution of leucofisetinidins in Table 3 by Roux & Evelyn (1960)]. Heartwoods containing very variable distributions of these compounds and of tannin components are also found.

Gleditsia triacanthos (Chadenson *et al.* 1955). Fustin from *Gleditsia japonica* (Mitsuno & Yoshizaki, 1957) forms a tetra-acetate of similar but slightly lower melting point (110–111°) than the corresponding derivatives of the enantiomorphs (cf. Table 1).

The enantiomorphism of (+)-fustin from *A. mollissima* and (−)-fustin from *Rhus cotinus* constitutes the second example of the presence of enantiomorphous flavonoid compounds in Nature. Previously (+)- and (−)-7:3′:4′-trihydroxyflavan-3:4-diols from *A. mollissima* and *Schinopsis quebracho-colorado* respectively were shown to be enantiomorphous by Clark-Lewis & Roux (1958, 1959). From recent work by Weinges (1958, 1959) the absolute configuration of (+)-fustin may be inferred. (+)-Fustin has the same absolute configuration (2:3-*trans*) as (+)-catechin and other naturally occurring dihydroflavonols (Mahesh & Seshardi, 1955; Whalley, 1956; Clark-Lewis & Korytnyk, 1958) and is tentatively represented as (2R:3R)-3:7:3′:4′-tetrahydroxyflavan-4-one (cf. Cahn, Ingold & Prelog, 1956; Birch, Clark-Lewis & Robertson, 1957). Similarly, (−)-fustin, shown to have the same configuration as (−)-catechin by Weinges (1959), may be designated as (2S:3S)-3:7:3′:4′-tetrahydroxyflavan-4-one. (−)-Fustin from *R. cotinus* has been hydrogenated to (−)-7:3′:4′-trihydroxyflavan-3:4-diol (Weinges, 1959), which occurs naturally in *Schinopsis* spp. (Roux, 1958; Freudenberg & Weinges, 1958; Roux & Evelyn, 1958). Thus (−)-fustin and (−)-leucofisetinidin form a group of hitherto unusual naturally occurring (2S)-flavan derivatives and also belong to a natural group of flavonoid compounds ('resorcinol series') unhydroxylated in the 5 position. The 5-deoxyflavanone, (−)-liquiritigenin, from *Glycyrrhiza glabra* although designated as (2S) (Arakawa & Nakazaki, 1960), has the same configuration at the 2 position as (+)-catechin and therefore does not belong to the above-mentioned group.

The conversion of (+)-fustin by hydrogenation with platinum oxide in methanol (method of Freudenberg & Roux, 1954) into (+)-7:3′:4′-trihydroxyflavan-3:4-diol, $[\alpha]_D^{25} + 36{\cdot}7°$, possibly represents the first synthesis of (+)-mollisacacidin [(+)-gleditsin] from fustin, as Keppler (1957) apparently used (±)-fustin from *R. glabra* and therefore obtained (±)-7:3′:4′-trihydroxyflavan-3:4-diol (cf. Roux & Freudenberg, 1958; Clark-Lewis & Roux, 1959). (+)-Fustin and (+)-mollisacacidin occurring in the heartwood of *Acacia mollissima* therefore have identical configurations at C-2 and C-3. Freudenberg & Weinges (1959) have drawn attention to a similar relationship between (−)-fustin and (−)-7:3′:4′-trihydroxyflavan-3:4-diol found in *R. cotinus*, and between (+)-dihydrobinetin and (+)-7:3′:4′:5-tetrahydroxyflavan-3:4-diol occurring in *Robinia pseudacacia*. Mitsuno & Yoshizaki's (1957) undesignated fustin accompanying (+)-gleditsin [(+)-7:3′:4′-trihydroxyflavan-3:4-diol] in *Gleditsia japonica* is evidently a pure enantiomer, and therefore probably a (+)-fustin identical with that from *A. mollissima*.

In black-wattle heartwood (+)-mollisacacidin occurs in highest concentration in the peripheral heartwood and decreases in concentration towards the centre (Table 2), whereas (+)-fustin, present in relatively low concentration in the peripheral heartwood, increases in concentration towards the centre. Visual observation of the quantitative chromatograms showed that fisetin, which is almost absent in the peripheral heartwood, increases progressively in concentration towards the centre. These distributions, together with the stereochemical and chemical interrelationships, suggest that (+)-7:3′:4′-trihydroxyflavan-3:4-diol is the primary body formed in biosynthesis, and that apart from condensation to polymeric leucofisetinidins, it undergoes interconversion into (+)-fustin and finally into fisetin during aging of the wood. Both condensation and interconversion could result from dehydrogenation mechanisms.

SUMMARY

1. (+)-Fustin and fisetin have been isolated from the heartwood of *Acacia mollissima*.

2. (+)-Fustin has been shown to be enantiomorphous with (−)-fustin from *Rhus cotinus*, whereas *Rhus glabra*, *Rhus succedanea* and *Rhus vernicifera* contain racemic mixtures of these enantiomorphs. The stereochemistry of these fustins is inferred from previous work.

3. (+)-Fustin was converted into (+)-7:3′:4′-trihydroxyflavan-3:4-diol [(+)-mollisacacidin] by hydrogenation.

4. The radial distributions of (+)-mollisacacidin, (+)-fustin and fisetin in wattle heartwood were estimated. The findings suggest that (+)-mollisacacidin is the primary precursor from which (+)-fustin and fisetin are formed by dehydrogenation.

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Biochem. J. (1960) **77**, 320

An Inborn Defect in the Metabolism of Tyrosine in Infants on a Normal Diet

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Levine and his coworkers (Levine, Marples & Gordon, 1939, 1941; Levine, Gordon & Marples, 1941; Levine, Dann & Marples, 1943) showed that premature, but not full-term, infants excrete *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyllactic acid if fed on a high protein diet, but both groups of infants excrete these compounds if given a 'loading' dose of tyrosine or phenylalanine. This was confirmed and extended by Woolf & Edmunds (1950), who showed that, after a 'loading' dose of tyrosine or phenylalanine, both premature and full-term infants exhibit a temporary hydroxyphenyluria followed by a return to normal values though the peak excretion is much higher in the premature group.

Ascorbic acid promptly cured the hydroxyphenyluria in these infants (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950) and it rarely appeared in an infant that had ever been fed with ascorbic acid. Adults or infants suffering from scurvy also showed hydroxyphenyluria when fed with large doses of tyrosine (Rogers & Gardner, 1949; Morris, Harpur & Goldbloom, 1950) and here again feeding with ascorbic acid cured the hydroxyphenyluria. Independently of the above work, Sealock & Silberstein (1939, 1940) had shown that scorbutic guinea pigs developed hydroxyphenyluria if they were fed with tyrosine. The effect of ascorbic acid on tyrosine metabolism by liver slices etc. *in vitro* has been reviewed by Knox (1955) (see also Zannoni & La Du, 1960).

Hydroxyphenyluria also occurs in advanced liver disease (Felix, Leonhardi & Glasenapp, 1951; L. I. Woolf, unpublished work); in this condition ascorbic acid has no effect.

Work *in vitro* on the foetal and neonatal development of enzymes concerned with the metabolism of phenylalanine and tyrosine has been carried out by Kretchmer, Levine, McNamara & Barnett (1956), Kretchmer & McNamara (1956), Reem & Kretchmer (1957), Kenney, Reem & Kretchmer (1958) and Auerbach & Waisman (1959).

During a survey of young infants in Cardiff to detect cases of phenylketonuria (Gibbs & Woolf, 1959), several infants were found whose urine gave a green colour with ferric chloride, but who were not phenylketonurics. As reported below, this has been found to be due to *p*-hydroxyphenylpyruvic acid and these urines have also been found

Table 1. *Details of the infants investigated*

D.M., Dried milk; B.F., breast-fed (figures in parentheses indicate the number of days that the infant was breast-fed after birth); —, not known.

Infant no.	Age (days)	Wt. (kg.)	Diet		Ascorbic acid supplement		Birth wt. (kg.)	Maturity at birth (weeks)
			Type	g./day	During pregnancy	After birth		
1	25 32	3·32 3·32	Cow's milk	610 (ml.)	Occasional	None	2·50	—
2	30	—	D.M. (B.F. 2)	37·2	Adequate	None	2·78	35½
3	38 47 68	—	D.M. (B.F. 2)	26·6 35·4	Adequate	From 1 month	2·04	—
4	54 66	—	D.M. (B.F. 7)	26·6	Adequate	From 6 weeks	1·68	37
5	26	3·49	D.M.	37·2	Adequate	From 1 month	3·04	38
6	37 41	— 3·14	D.M. (B.F. 8)	31·8	None	From 8 days	2·40	34
7	38 41	3·51 3·68	D.M.	31·8	—	From birth	2·36	38
8	30 33	— 4·31	B.F. + mixed	—	Adequate	From 1 month	3·06	40½
9	48	4·76	D.M. (B.F. 3)	35·4	Adequate	From 1 month	2·42	36
10	22	4·25	D.M. (B.F. 28)	32	Adequate	None	3·74	41½
11	39	4·65	D.M. (B.F. 14)	44	Very little	From 1 month	2·95	40
12	24 30 44	3·86	D.M.	48	Irregular	From 1 month	3·44	42½
13	26	3·97	D.M. (B.F. 9)	—	Adequate	From 1 month	3·12	40
14	35 40	3·70 3·70	D.M. (B.F. 2)	37·2	None	From 4 weeks	2·61	39½

to contain large amounts of *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid. This hydroxyphenyluria is discussed in relation to the maturation of enzyme systems, to diet and to prematurity.

MATERIALS

Urine specimens from 1276 infants, aged between 3 and 8 weeks, were examined. In each case the mother collected the produce of a single voiding at home in a clean bottle containing a little chlorbutol as preservative. Fourteen infants passed urine giving a green colour with ferric chloride (excluding the one phenylketonuric detected). These specimens were investigated more fully and efforts were made to get further urine specimens from these infants. Details of the infants are given in Table 1.

Several preparations of dried milk (half-cream, full-cream, National or proprietary) were used. Only ascorbic acid taken in the form of orange juice, rose-hip syrup or some proprietary preparation is shown in Table 1. Ascorbic acid from other sources (e.g. breast milk or potatoes) is ignored. In column 9 the 'maturity' is the interval between the last menstrual period and birth, where known.

p-Hydroxyphenyl-lactic acid was prepared by reducing *p*-hydroxyphenylpyruvic acid (Sigma Chemical Co.) with sodium amalgam in acid solution. The material obtained from an ethereal extract was crystallized from ethyl acetate–benzene. The white crystals were dried over P_2O_5, KOH and paraffin wax *in vacuo*. The material gave a single spot in the expected position on paper chromatograms giving the expected reactions with diazotized sulphanilamide and *p*-nitroaniline.

METHODS

A 5% (w/v) solution of $FeCl_3,6H_2O$ in water was added drop by drop to a portion of the urine. A Phenistix strip (Ames Co., Nuffield House, London, W. 1) was dipped into a second portion. A saturated solution of 2:4-dinitrophenylhydrazine in 2N-HCl was, in most cases, added to a third portion.

Some of the specimens were so small in volume that not all the investigations could be carried out. Tyrosine (including tyramine), free phenols, conjugated phenols and *p*-hydroxyphenylpyruvic acid were estimated as before (Woolf & Edmunds, 1950; Medes, 1932). The ethereal extract of the acidified urine was re-extracted with sodium

bicarbonate solution and the phenolic acids were examined by paper chromatography. Girard's reagent P (Girard & Sandulesco, 1936) was added to a fresh portion of the urine, the solution was left for 10 min., brought to pH 2 and extracted six times with an equal volume of ether each time; the pooled ethereal extracts were re-extracted with sodium bicarbonate solution and a portion of this was tested for compounds capable of reducing Brigg's reagent (strongly acid phosphomolybdate) (cf. Berry & Woolf, 1952). Other portions were used for paper chromatography of non-ketonic phenolic acids.

Paper chromatography was used to examine the urines for amino acids (Woolf & Norman, 1957), sugars, phenolic substances (one- and two-dimensional, for solvents cf. Armstrong, Shaw & Wall, 1956) and indole derivatives (one- and two-dimensional, Jepson, 1955). The spray reagent used for phenols was generally diazotized sulphanilamide (Block, Durrum & Zweig, 1958), but in some cases duplicate chromatograms were sprayed with a *p*-nitrobenzenediazonium reagent prepared as follows: an aqueous 0·5% solution of Brentamine Fast Red GG (Imperial Chemical Industries Ltd.) was mixed with an equal volume of aqueous 50% (w/v) sodium acetate solution, the chromatogram was immediately sprayed with the mixture and resprayed, 1 min. later, with half-saturated sodium carbonate solution (cf. Harvey & Penketh, 1957).

Two specimens of urine were hydrolysed by refluxing with 6N-HCl for 16 hr. and the amino acids present were compared with the unhydrolysed specimens by paper chromatography. The specimen from infant no. 3, 47 days, was ultrafiltered through Visking regenerated cellulose tubing, and the unfiltered residue, about three times concentrated with respect to colloids, was hydrolysed and chromatographed as described above. The ultrafiltrate was passed through a column (10 cm. × 1 cm. diam.) of Zeo-Karb 225 (a sulphonated polystyrene resin, about 8% cross-linked, H form, beads approx. 0·5 mm. diam.) and the material unadsorbed by the acid resin was passed through a column (10 cm. × 1 cm. diam.) of Amberlite IR-45 (a weakly basic resin, OH form, beads approx. 0·5 mm. diam.). The ammonia eluate of the Zeo-Karb column was divided into two, and one half was hydrolysed as described above; both hydrolysed and unhydrolysed fractions were then chromatographed for amino acids. The N-H_2SO_4 eluate of the Amberlite IR-45 column and the material adsorbed by neither resin were hydrolysed and chromatographed as described above.

RESULTS

The results of the ferric chloride and 2:4-dinitrophenylhydrazine reactions, and the concentrations of hydroxyphenyl compounds, are given in Table 2. All the specimens either gave no reaction with Phenistix or produced a fleeting green colour. After paper chromatography in butan-1-ol–acetic acid–water (4:1:1), *p*-hydroxyphenylpyruvic acid, unlike *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid, which give non-fluorescent red spots, gave only a very faint brown spot with the diazotized sulphanilamide reagent but the spot fluoresced brilliant blue in ultraviolet light (3650 Å). *p*-Hydroxyphenylpyruvic acid ran to the same position as *p*-hydroxyphenyl-lactic acid but both could be detected, if present together, with the diazotized sulphanilamide reagent by reading in visible and in ultraviolet light. On two-dimensional chromatograms any *p*-hydroxyphenylpyruvic acid present was generally destroyed. Apart from *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid, tyrosine was generally the only phenolic substance present in abnormal amounts. However, four specimens (from infants nos. 1, 5 and 14) contained, in addition to these three substances, considerable amounts of an unidentified substance, of high R_F both in butanol–acetic acid and in propan-2-ol–ammonia, that gave a yellow colour with the diazotized sulphanilamide reagent and a red colour with the *p*-nitrobenzenediazonium reagent. Incubation of *p*-hydroxyphenylpyruvic acid solution at various temperatures and pH values, with and without urea and ammonium sulphate, failed to produce this substance, but produced varying amounts of *p*-hydroxyphenylacetic acid. In 4 hr. at room temperature, most *p*-hydroxyphenylacetic acid was formed at pH 9·0–9·6; less was formed at pH 6·4 and none at pH 3·2. The amount of *p*-hydroxyphenylacetic acid found in the urines was about one-tenth of the amount of *p*-hydroxyphenyl-lactic acid. The ethereal extracts of the acidified urine contained *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid, but in the presence of Girard's reagent P only *p*-hydroxyphenyl-lactic acid and *p*-hydroxyphenylacetic acid were extractable, giving further evidence of the identity of the keto acid.

No abnormal indole derivatives were found in the 11 specimens examined. However, the indican content was low or very low in all and none could be detected in two (cf. Woolf & Edmunds, 1950).

The sugars in the five specimens examined were normal (Woolf & Norman, 1957), the galactose content ranging from a trace to 12 mg./100 ml., the fructose from 0 to 10 mg./100 ml. and the glucose from 0 to 10 mg./100 ml. No other sugars were detected.

Protein was absent from all the urine specimens. Of the ten specimens examined semi-quantitatively for amino acids, six had tyrosine present in amounts greatly above the normal, in two it was normal or slightly raised in amount, in one it was almost absent and in one it could not be detected at all. Taurine was present in very large amount in the last two specimens but was absent, or present in very low concentration, in the other eight. The other amino acids, including phenylalanine, were normal for infants of this age (Woolf & Norman, 1957). Homogentisic acid, which gives a characteristic brown spot, and 3:4-dihydroxyphenylalanine were not detected in any specimen. No

Table 2. *Analysis of the urine specimens*

+, Indicates formation of a precipitate with 2:4-dinitrophenylhydrazine or of a green colour with ferric chloride.

Infant no.	Age (days)	$FeCl_3$ reaction	2:4-Dinitrophenylhydrazine reaction	*p*-Hydroxyphenylpyruvic acid (mg./100 ml.)	Free phenols (as tyrosine) (mg./100 ml.)	Conjugated phenols (as tyrosine) (mg./100 ml.)	Tyrosine (mg./100 ml.)	Creatinine (mg./100 ml.)
1	25	+	+	25·7	189	33	28	36
	32	+	+	11·8	91	10	32	17
2	30	+	+	22	215	58	18	.
	79	−	.	.	.	.	.	.
3	38	+	.	.	.	.	.	.
	47	+	+	12	97	11	7	8
	68	+	+	3·9	44	7	11	21
	103	−	.	.	.	.	.	.
4	54	+	+	9·2	66	10	15	15
	66	+	+	6	19	35	5	10
	84	−	.	.	.	.	.	.
5	26	+	+	7·5	51	23	23	42
	47	−	.	.	.	.	.	.
6	37	+	.	.	.	.	.	.
	41	−	−	0	3	2	0	17
7	38	+	+	19·2	116	63	2	54
	41	−	−	3·3	10	18	6	10
8	30	+	.	.	.	.	.	.
	33	−	−	.	.	.	.	12
9	38	+	.	.	.	.	.	.
	48	+	+	12·4	91	24	18	25
	66	−	.	.	.	.	.	.
10	22	+	−	.	.	.	.	.
	36	−	.	.	.	.	.	.
11	39	+	+	9·0	37	28	2	11
	53	+	.	.	.	.	.	.
	60	−	.	.	.	.	.	.
12	24	+	.	.	.	.	.	.
	30	+	.	.	.	.	.	.
	44	−	.	.	.	.	.	.
13	26	+	+	4·5	63	14	4	23
	40	−	.	.	.	.	.	.
14	35	+	+	24·4	152	52	39	55
	40	−	.	.	.	.	.	.

spot in the position of tyrosine *O*-sulphate was found in any specimen of urine.

In the two specimens examined (from infant no. 3, 47 days, and infant no. 4, 66 days) the conjugated amino acids released on acid hydrolysis were normal; in particular no extra tyrosine was released from the second and the tyrosine content in the first rose on hydrolysis from about 0·07 mg./ml. to about 0·1 mg./ml. On fractionation of this specimen, the residue from ultrafiltration released on hydrolysis no more tyrosine than did the original urine. Acid hydrolysis of the ammonia eluate of the Zeo-Karb released as much tyrosine, within experimental error, as was released from the original urine. The material adsorbed by the basic resin released only a trace of tyrosine on hydrolysis, but none could be detected in the hydrolysate of the unadsorbed fraction.

DISCUSSION

Where the enzyme system converting *p*-hydroxyphenylpyruvic acid into homogentisic acid (Knox & LeMay-Knox, 1951) is functioning poorly, or not at all, dietary phenylalanine and tyrosine can produce hydroxyphenyluria, especially if a high 'loading' dose is given. This can be due to absence of a cofactor such as ascorbic acid, to immaturity or to damage to the liver cells.

Zannoni & La Du (1960) have shown that the liver of scorbutic guinea pigs contains as much *p*-hydroxyphenylpyruvic acid oxidase as in non-scorbutic guinea pigs, but that in the scorbutic animals this enzyme is inactivated by excess of its substrate, both *in vivo* and *in vitro*, and that ascorbic acid prevents or reverses this inactivation. A similar mechanism would explain the results

obtained by Woolf & Edmunds (1950) in premature infants. Ascorbic acid appears to act by keeping some other compound, possibly the true coenzyme, in an active state (Zannoni & La Du, 1959, 1960).

It seems probable that infants store ascorbic acid during the last few weeks of gestation (Park, Guild, Jackson & Bond, 1935; Toverud, 1935; Steinebrei, 1939). The induced hydroxyphenyluria of prematurity (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950) is probably due to a simple lack of ascorbic acid, as shown by the extremely rapid reversion to normal on giving this vitamin. In the cases reported here, seven had received supplements of ascorbic acid (according to the mothers' accounts) for some time before passing the hydroxyphenyluric urine. Ten of the infants were breast-fed for a time, which might provide an additional source of ascorbic acid. None of the mothers showed signs of ascorbic acid deficiency during pregnancy, and seven of them took supplementary ascorbic acid. It seems improbable that these infants lacked ascorbic acid, and in any case deficiency of this vitamin will not produce hydroxyphenyluria unless the premature infant or scorbutic infant or adult is also given a high-protein diet or a 'loading' dose of phenylalanine or tyrosine (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950; Rogers & Gardner, 1949; Morris *et al.* 1950).

None of the infants' diets reached the level of 5 g. of protein/kg. body wt./day (see Table 1), and their weight gains were consistent with this level of feeding (cf. Levin, Mackay, Neill, Oberholzer & Whitehead, 1959). This level was found by Levine *et al.* to be generally necessary for establishing hydroxyphenyluria in premature infants.

In a previous study (Woolf & Edmunds, 1950) small premature infants who had never received ascorbic acid were fed with a dose of 4 g. of tyrosine, about seven times their normal daily intake. Their mean birth weight was 1·88 kg., the gestation period ranged from 28 to 37 weeks (mean 33 weeks), and the sum of age and gestation was between 31½ and 38½ weeks (mean 35½ weeks). The concentrations of *p*-hydroxyphenylpyruvic acid and *p*-hydroxyphenyl-lactic acid in their urine after the test dose were no higher than in the present group of infants, who were more mature on each of the three criteria of birth weight, gestation period and age plus gestation (Table 1). About one-quarter of the dietary phenylalanine and tyrosine was excreted as hydroxyphenyl derivatives by the present group of infants (calculated from the creatinine concentrations). The infants studied by Woolf & Edmunds (1950) were plainly metabolizing *p*-hydroxyphenylpyruvic acid at a far greater rate, but in both groups either some *p*-hydroxyphenylpyruvic oxidase was present or some alternative pathway of tyrosine metabolism was being used.

Kretchmer *et al.* (1956) reported that liver homogenates from the majority of very premature infants lacked the enzymes necessary for oxidizing tyrosine to maleylacetoacetic acid and that, unlike adult-liver homogenates, the addition of ascorbic acid had little effect. The hydroxyphenyluria reported here is, we suggest, also due to the failure to synthesize an enzyme, either completely or in sufficient amount, even though the infants were not nearly so premature as those investigated by Kretchmer *et al.* (1956), or even as premature as infants in whom this ability had already appeared (Levine *et al.* 1939, 1941, 1943; Woolf & Edmunds, 1950). In spite of this immaturity of one enzyme system the children were well, with no evidence of jaundice or liver disease and, in particular, phenylalanine hydroxylase and the enzymes concerned with tryptophan metabolism and galactose metabolism appear to have developed normally. Tyrosine transaminase was evidently active since *p*-hydroxyphenylpyruvic acid was formed, but it is uncertain whether the high excretion of tyrosine reflected a lower transaminase activity than normal or the accumulation of *p*-hydroxyphenylpyruvic acid leading to increased reconversion into tyrosine.

This enzyme defect can be compared with tyrosinosis, as described by Medes (1932), and the inborn errors of metabolism. Unlike these, however, the hydroxyphenyluria in these infants was transient, vanishing remarkably abruptly (Table 2). We suggest that this enzyme system suddenly becomes active at an age which varies from individual to individual with a continuous distribution from (say) 5 to 12 months from conception. Thereafter its activity probably increases more gradually since Kretchmer *et al.* (1956) found that adult liver had, weight for weight, about four times the tyrosine-oxidizing activity of normal full-term liver. It would seem that a person can live and thrive for a time without a normal amount of this enzyme. If this were not so the incidence of hydroxyphenyluria would surely be much lower since selection would operate against it unless there were some compensating genetic advantage.

Five of these 14 infants weighed less than 2·5 kg. at birth, but only 7·5 % of infants born in Cardiff during the period were premature on a weight basis. This indicates some correlation between prematurity and immaturity of the enzyme system for metabolizing tyrosine, but prematurity is neither a necessary nor a sufficient cause of the hydroxyphenyluria. The incidence of hydroxyphenyluria, even at an age greater than 3 weeks, may well be higher than is suggested by 14 cases out of 1276 infants tested, since the ferric chloride reaction is

not very reliable for detecting *p*-hydroxyphenylpyruvic acid, being very sensitive to variation in experimental conditions, and mild cases, if they occur, would probably excrete very little of the keto acid, the major metabolite being *p*-hydroxyphenyl-lactic acid (cf. Levine *et al.* 1941).

p-Hydroxyphenylacetic acid has not previously been reported to occur in hydroxyphenyluric urine. It could arise *in vivo* from *p*-hydroxyphenylpyruvic acid by oxidative decarboxylation (Kirberger & Bücher, 1952), or it could be an artifact formed after the urine is voided. As noted above, *p*-hydroxyphenylpyruvic acid solution gives rise to *p*-hydroxyphenylacetic acid on standing at neutral or alkaline pH.

The absence of 3:4-dihydroxyphenylalanine from these urines is a further difference from tyrosinosis (Medes, 1932). In one of the two specimens examined, a considerable fraction of the total tyrosine was in conjugated form, as found before (Woolf & Edmunds, 1950). The tyrosine conjugate was not appreciably held back by ultrafiltration, and therefore it is not a protein or high-molecular-weight peptide. Almost the whole of the tyrosine conjugate was adsorbed by the strong acid resin; it follows that it had basic properties, unlike tyrosine *O*-sulphate, *N*-acyl derivatives of tyrosine or tyrosine glucuronide. These strongly acid conjugates are adsorbed by basic resins, but they must have been present only in traces in the urine. The bulk of the conjugated tyrosine is probably present as a peptide (or peptides) of low molecular weight. Tyrosine *O*-sulphate has been found in human urine by Tallan, Bella, Stein & Moore (1955), who suggest that it may be a conjugate formed from tyrosine as other phenols form ethereal sulphates. However, no tyrosine *O*-sulphate was found in any of the urines examined by us, including the specimen which contained much bound tyrosine. Although the method we used would not detect very small amounts of tyrosine *O*-sulphate, such as are present in normal adult urine, it is clear that at most only a small fraction of the total tyrosine was present as the ethereal sulphate, in spite of the very large amounts of tyrosine that must have accumulated in these infants and that were being excreted in free and bound forms. This is in accordance with the work of Dodgson, Rose & Tudball (1959) and of Grimes (1959), who showed that tyrosine is not sulphated by various tissue preparations *in vitro*.

The absence of indolyl-lactic acid and indolylacetic acid from the urine can be contrasted with the findings in phenylketonuria (Armstrong & Robinson, 1954) and 'maple-syrup urine' disease (Mackenzie & Woolf, 1959), other conditions in which large amounts of an α-oxo acid accumulate in the body. It suggests that *p*-hydroxyphenylpyruvic acid may have less effect on general metabolism than some other α-oxo acids, since tryptophan metabolism is not interfered with, and this may, perhaps, be correlated with the good health of the infants reported here.

SUMMARY

1. Fourteen infants, out of 1276 tested, were found to excrete large amounts of *p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyl-lactic acid and tyrosine. *p*-Hydroxyphenylacetic acid was present in the urine but may be an artifact. Tyrosine *O*-sulphate was not detected. A variable proportion of the tyrosine was conjugated as a low-molecular-weight compound with basic properties.

2. In contrast with previous reports of hydroxyphenyluria, these infants were fed on a normal diet with only a moderate protein intake and (in seven cases) supplementary ascorbic acid. They were well, with no evidence of liver dysfunction. Some were premature and some full-term, and they were aged up to 68 days. The hydroxyphenyluria ceased abruptly after a time without any treatment.

3. It is suggested that these infants lacked an enzyme concerned with oxidizing *p*-hydroxyphenylpyruvic acid to homogentisic acid and that the ability to carry out this reaction first appears at different absolute ages in different individuals.

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Biochem. J. (1960) **77**, 326

A Tissue Homogenizer

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For the separation and isolation of the particulate fractions existing within cells, the cells must be disrupted. In studies of the action of toxic substances upon mitochondria it was important that we should use a method of homogenization which was both efficient and reproducible. It was also essential that if the homogenizer was broken it could be readily replaced by one with an identical performance. The above criteria are not all fulfilled by previously described homogenizers.

The homogenizer described has been designed on the general principles of that of Potter & Elvehjem (1936) and the constructional details are given in Fig. 1. A Perspex pestle rotates in a smooth precision-bore glass tube. Since Perspex is easy to machine a precise gap between pestle and mortar may be readily obtained. Instead of the usual spherical end, the end of the pestle is a cone of 90° angle; this fits accurately into the base cavity and also allows the shape to be accurately repeated when required.

It is essential to use a motor with sufficient power ($\frac{1}{4}$ h.p.) to prevent slowing of the pestle when the tissue and fluid is being forced through the gap between the pestle and mortar. Under these conditions the gap between pestle and mortar is likely to remain constant, thus maintaining constant the shearing forces in the fluid. The lack of contact between the two surfaces is indicated by the very slight wear in the pestle; after use for 18 months the diameter of the pestle had decreased by only 0·001 in. at the bottom and less at the top.

For the preparation of mitochondria the pestle has been rotated at 1100 rev./min. and the gap between the pestle and mortar varied for different tissues (0·01 in. for liver and 0·005 in. for brain). The properties of the liver and brain mitochondria so prepared have been previously described (Aldridge, 1957, 1958; Aldridge & Parker, 1960). Preliminary experiments have shown that with a gap of 0·005 in. mitochondria may be isolated from rat kidney. The oxidation of pyruvate and fumarate by this preparation is stimulated threefold by 2:4-dinitrophenol and potato apyrase and has an activity of 300 μl. of O_2/mg. of protein/hr. (for experimental details cf. Aldridge, 1958). Heart mitochondria could only be isolated (though in poor yield) when the tissue had been chopped (McIlwain & Buddle, 1953) before homogenization with a gap of 0·005 in. Such preparations were stimulated

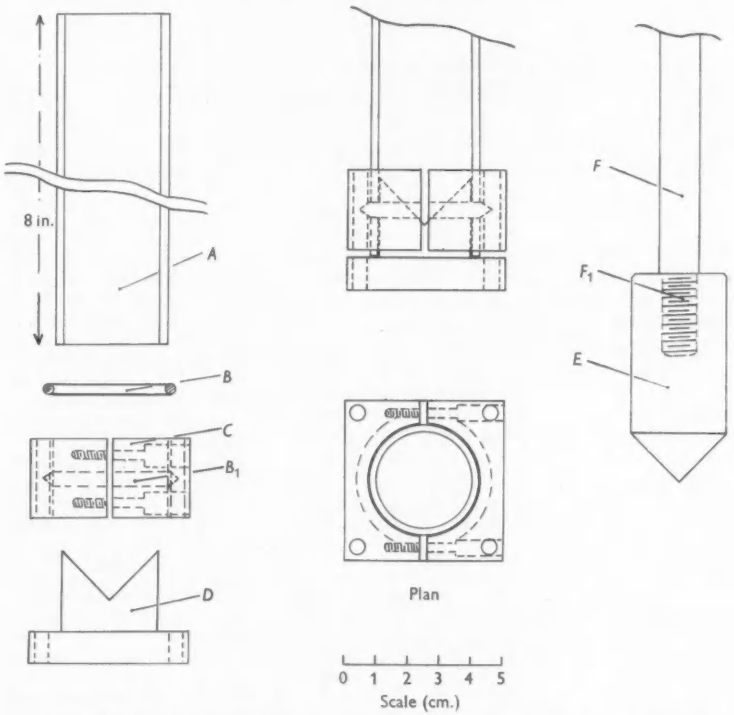

Fig. 1. Contructional details of the tissue homogenizer. *A*, Glass tube (Veridia tubing, manufactured by Chance Bros. Ltd.) with 30 mm. bore and 2 mm. wall thickness. *B*, Rubber O ring (Edwards High Vacuum Ltd., catalogue no. OR. 17). *C*, Two mated Perspex blocks, grooved as shown (B_1) to hold the rubber O ring and clamped just sufficiently to the glass tube to take the pressure of the rotating pestle. *D*, Perspex base, turned and shaped to the same angle (90°) as the Perspex pestle to be a sliding fit in the Veridia tubing; this is cemented to the glass tube with Bostik D and held in position by screws to the Perspex block *C*. *E*, Perspex block machined to shape with the necessary clearance between pestle and tubing. *F*, Stainless-steel rod (0·5 in. diam.) turned down at one end (F_1) and threaded ($\frac{7}{16}$ in. B.S.F.) to fit the pestle.

1·5–2·0 times by 2:4-dinitrophenol and apyrase and had an oxidative activity of 400–500 μl. of O_2/mg. of protein/hr.

The design of this homogenizer is in principle very similar to the rotating concentric-cylinder apparatus for the determination of viscosity. The relation between the shearing forces in the fluid, the radii of the cylinders and the angular velocity is known when the angular velocity is low (Poynting, Thomson & Todd, 1947). However, for homogenization, the angular velocity is high and the flow turbulent. Under these conditions it is impossible to calculate the shearing forces. It is therefore necessary with homogenizers of different dimensions to test empirically the best combination of gap and speed of rotation of the pestle. However, it has been our experience that changes in speed of rotation produce bigger changes in the proportion of cells broken than do changes in the gap between pestle and mortar.

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Biochem. J. (1960) **77**, 328

Kinetic Studies on the Reaction between Native Globin and Haem Derivatives

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It was reported by Hill & Holden (1926), and confirmed by later workers, that the reaction between haematin and globin is rapid, but no numerical results appear to have been published. The reaction between peroxidase apoprotein and haematin has, however, been examined (Theorell & Maehly, 1950). It was slow, with a half-time of the order of minutes; the results with different preparations showed variations up to seven-fold in the rate.

Rossi-Fanelli, Antonini & Caputo (1958) have obtained globin preparations which on reaction with haematin yield haemoglobin derivatives with functional properties indistinguishable from those of the natural protein (Rossi-Fanelli & Antonini, 1959; Antonini & Gibson, 1960). These authors have suggested that on recombination the haematin molecules return to just the same positions as those occupied in the natural protein. It is of some interest to know how rapidly this reaction can occur, as it bears on the accessibility of the haem-binding sites of the globin, though, of course, kinetic data alone cannot serve to locate these in relation to the remainder of the molecule.

The rate of reaction of native human globin with haematin, carboxyhaem and monocyanide haem has been measured under several experimental conditions. Parallel experiments have also been carried out with native human-myoglobin globin. A preliminary account of some of these experiments has already been presented by Gibson, Rossi-Fanelli & Antonini (1959).

EXPERIMENTAL

Materials

Potassium cyanide was of analytical quality (British Drug Houses Ltd.), sodium dithionite was Manox brand (supplied by Hardman and Holden, Miles Platting, Manchester).

Experiments at pH 9·1 were carried out with a borate buffer prepared by dissolving 1·9 g. of $Na_2B_4O_7,10H_2O$ in 100 ml. of water. Buffer of pH 10·6 was prepared according to Kolthoff (1925).

Haematin solutions were prepared by dissolving crystalline proto- or meso-haemin in 0·01 N-NaOH to give a solution of about 1 mM. This solution was further diluted as required with buffer to give the working solution.

Carboxyhaem solutions were prepared by reducing the necessary volume of stock haematin solution with a few crumbs of sodium dithionite (about 0·5 mg./ml.) and diluting with deoxygenated buffer which had been equilibrated with a suitable partial pressure (usually between 100 and 200 mm. Hg) of carbon monoxide.

Dicyanide haem solutions were prepared by dissolving the required weight of KCN in borate buffer, adding 0·5 mg. of sodium dithionite/ml., and then introducing the volume of stock haematin solution necessary to give the appropriate final concentration of dicyanide haem.

Myoglobin globin was prepared from crystalline human myoglobin by the method of Theorell & Akeson (1955).

Other materials and buffers were as described by Antonini & Gibson (1960).

Methods

Stopped-flow kinetic determinations were carried out with the apparatus of Gibson (1954). For many experiments with low concentrations of haematin and globin, a special observation tube was used which gave a light-path length of 2 cm. in place of the normal 2 mm. tube. This is constructed entirely of stainless steel except for the two acrylic resin plugs (see Fig. 1), through which the light beam enters and leaves. The observation tube itself is surrounded by a water-jacket to control the temperature and the whole assembly can be fitted into the stopped-flow apparatus interchangeably with the ordinary observation tube. The volume of fluid observed is 63 $mm.^3$, which, with the flow rates used, corresponds to a time difference of about 3 msec. between the oldest and newest portions of mixture being studied in any single stopped-flow determination. Control experiments in which myoglobin was allowed to react with different concentrations of carbon monoxide showed that the mean delay between mixing of the reagents and their arrival in the observation tube was 3 msec.

The reactions were followed spectrophotometrically with monochromatic light from a Hilger D. 246 monochromator.

The other procedures required were carried out as described by Antonini & Gibson (1960).

Concentrations of globin are stated in terms of a molecular weight of 16 500. The particle size was in many cases a multiple of this value (Rossi-Fanelli *et al.* 1958).

RESULTS

Combination of haematins with globins

The time course of the early part of the reaction of native human globin from haemoglobin with protohaematin is shown in Fig. 2. The concentration of haematin was 33 μM, and the globin concentration varied from 16 to 132 μM. Except for

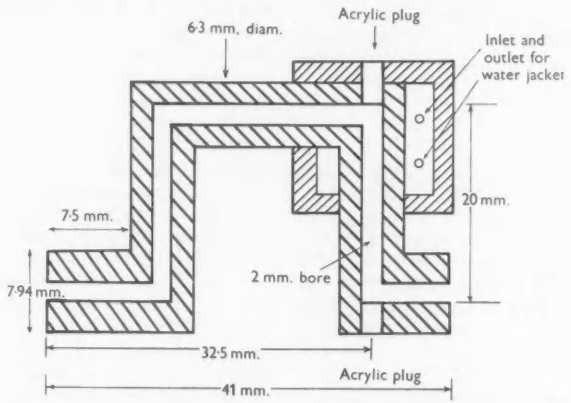

Fig. 1. Sectional diagram of observation tube for stopped-flow apparatus with 2 cm. light-path length.

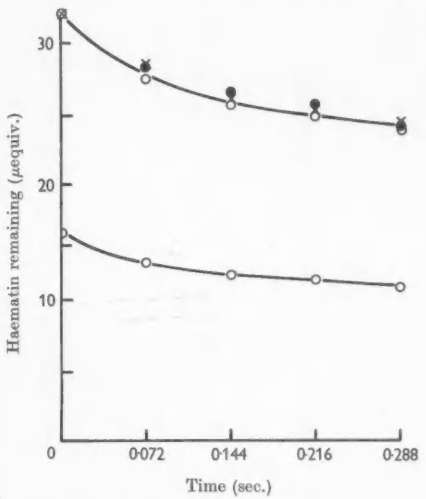

Fig. 2. Reaction of protohaematin with human globin. Haematin, 33 μM after mixing. Temp. 21°; 0·1 M-phosphate buffer, pH 7·1. Observations were made at 406 mμ, light path 2·38 mm. Globin: ○ (lower curve), 17 μequiv.; ○ (upper curve), 33 μequiv.; ×, 66 μequiv.; ●, 132 μequiv.

the lowest globin concentration, where only 0·5 equiv. of globin was added, the experimental curves do not vary significantly as the globin concentration is increased. The reaction is biphasic, about 20 % of the total taking place in the first 0·15 sec., whereas about 15 sec. is required for 90 % completion.

Qualitatively, the biphasic time course and the lack of effect of change in globin concentration may be explained by supposing that the results depend chiefly on the behaviour of the haematin. Shack & Clark (1947) have shown that haematin in aqueous buffer solutions is polydisperse, with particle weights ranging up to many thousands, representing aggregates of up to 100 haematin molecules. The initial rapid change of extinction may be explained as due to the combination of monomeric haematin with globin, and the slower long-continuing change as due to the dispersal of haematin aggregates followed by the combination of the dispersed haematin with other specific sites on the globin molecule. Although no definite value for the velocity constant of the haematin + globin reaction can be given, a lower limit can be set, at 5×10^4 M^{-1} sec.$^{-1}$.

Experiments similar to those shown in Fig. 2 have been carried out on the reaction of haemoglobin globin with meso- and deutero-haematin, and in addition the reaction of myoglobin globin with proto-, meso- and deutero-haematin has been examined. These results are summarized in Fig. 3, which shows that the rates with meso- and deutero-haematin are of the same general order as those for haemoglobin globin combining with protohaematin. The reaction of myoglobin globin with protohaematin is not shown in Fig. 3: in an experiment in which haemoglobin globin and myoglobin globin were compared side by side the progress curves for the two proteins agreed within experimental error and would lie closely upon the curve in Fig. 3 representing the reaction of mesohaematin with haemoglobin globin.

The rate of reaction observed in different experiments, carried out under apparently similar conditions, varied up to threefold. These differences,

which are similar to those found by Theorell & Maehly (1950) with peroxidase, may be due to variations in the degree of polymerization of the haematin solutions used on different days or at different times after preparation.

Combination of haem with globin

Equilibrium studies by Shack & Clark (1947) have suggested that haem (Fe^{2+}) in aqueous solution is dimeric, and it was hoped that the reactions of this derivative with globin might be more suitable for kinetic analysis.

The reaction of 33 μM-haem reduced by the addition of 1 mg. of sodium dithionite/ml. with 1 equivalent of haemoglobin globin gave an initial velocity constant of 3×10^4 M^{-1} sec.$^{-1}$ at 17° and pH 7·1.

The rate of reaction did not show the marked falling off which is so prominent in the records of Figs. 2 and 3.

Combination of carboxyhaem with globin

In view of the rather more satisfactory results obtained with free haem the reaction with carboxyhaem was examined next. Hill (1926) concluded that this compound has the formula CO–H–X, where H represents the haem nucleus and X may be either another molecule of the complex or a molecule of water. Since then it has generally been assumed to be monomeric in aqueous solution, a belief confirmed by Smith's (1959) spectrophotometric observations.

It was expected that if carboxyhaem is indeed monomeric the reaction rate observed might more nearly represent the true kinetic possibilities of haem derivatives in combining with globin than that observed with haematin and haem.

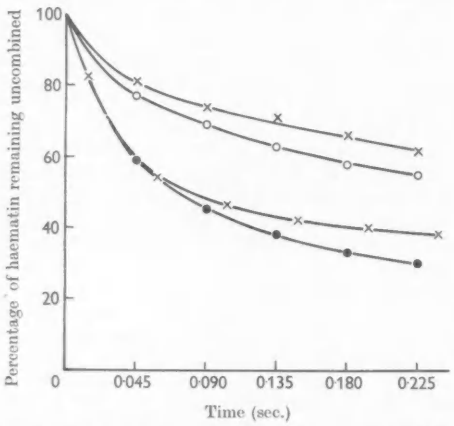

Fig. 3. Mesohaematin combining with myoglobin globin and haemoglobin globin. Haemoglobin globin 33 μM, myoglobin globin 33 μM, mesohaematin 33 μM. Wavelength of observation 397 mμ; 21°; phosphate buffer, pH 7·1; light path 2·38 mm. ○, Myoglobin globin; ×, haemoglobin globin. Deuterohaematin combining with myglobin globin and haemoglobin globin. All conditions were as for mesohaematin. ●, Myoglobin globin; ×, haemoglobin globin.

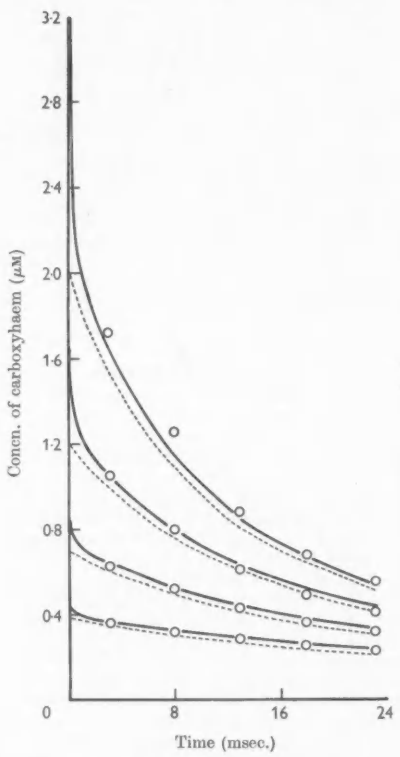

Fig. 4. Combination of carboxyhaem with human haemoglobin globin. Four concentrations of carboxyhaem and globin were used, giving concentrations after mixing of 3·2, 1·64, 0·84 and 0·43 μM of each. The reagents were dissolved in borate buffer, pH 9·1, equilibrated with a partial pressure of 200 mm. carbon monoxide in nitrogen. The reaction was followed at 421 mμ with a light-path length of 2 cm. The observations are shown as points; the continuous lines were computed from equations (1) and (2) by numerical methods and broken lines were calculated from equation (8) with the values derived from Fig. 7. [Broken lines start from lower concentrations of reagents because equation (8) supposes that an equilibrium concentration of haem–globin complex is present immediately upon mixing.] Values of the constants used in obtaining the continuous lines were: $k_1 = 4{\cdot}75 \times 10^8$ M^{-1} sec.$^{-1}$, $k_2 = 1\,750$ sec.$^{-1}$, $k_3 = 375$ sec.$^{-1}$.

The reaction observed with carboxyhaem was rapid, and an example of the results is given in Fig. 4, where four different sets of initial concentrations of carboxyhaem and globin were used. This experiment showed that the reaction was apparently second order with the lowest concentration examined, and gave a value for the rate constant of 8×10^4 M^{-1}sec.$^{-1}$. This value is among the highest ever to be obtained directly by flow methods, and the reaction is one of the most rapid known involving biological materials. Closer examination of the results has shown that the reaction is complex, and a detailed reaction scheme is proposed in a later section. In order to allow comparisons between experiments carried out under different conditions, the results have been calculated as if the reaction were second order, assuming that the concentrations of reactants were those put in the apparatus rather than those calculated from the observed changes in extinction. The reasons for this appear below.

Effect of pH *and of temperature.* A detailed study of the effect of pH has not yet been made, but experiments have been carried out at pH 7·1 and 9·1. The results, together with those giving the effect of temperature on the rate of the reaction, are summarized in Fig. 5, which shows that the observed rate is doubled by raising the pH to 9·1. The temperature coefficient is similar at both pH values and corresponds to an activation energy of about 11 kcal.

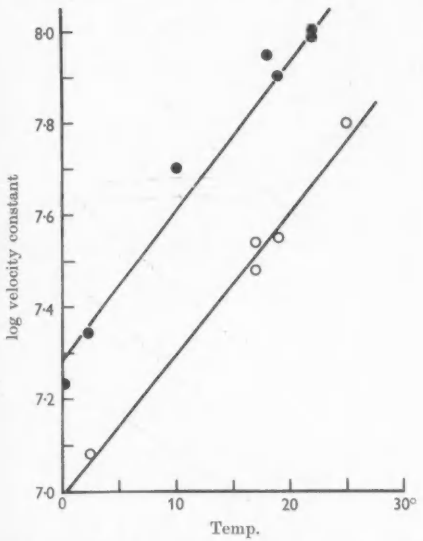

Fig. 5. Effect of temperature and pH on the observed rate of combination of carboxyhaem with globin. The ordinate gives the logarithm of the apparent second-order velocity coefficient. ●, Observations made with borate buffer, pH 9·1; ○, observations made with phosphate buffer, pH 7·1. Observations were made at 421 mμ, 2 cm. light-path length. Concentrations of haem and globin after mixing were 1 μM each.

Influence of other factors on the observed reaction

Wavelength of observation beam. The greater number of observations have been made with light of 421 mμ, corresponding to the maximum light absorption of carboxyhaemoglobin. In a few cases the reaction has also been followed by the changes taking place at 407 mμ, the absorption maximum of carboxyhaem. The observed rates have agreed well as measured at the two wavelengths.

Effect of salt concentration. The rate of combination of carboxyhaem with exhaustively dialysed globin dissolved in distilled water was measured. The haemin was dissolved in a minimum of NaOH solution and reduced under anaerobic conditions with a small excess of dithionite. The quantities of salts used were calculated to give a final ionic strength of less than 1×10^{-4}. After the determination of the combination rate an oxygen-free saturated solution of $(NH_4)_2SO_4$ was added to each reagent in quantities sufficient to give a final concentration of 0·1 M. [$(NH_4)_2SO_4$ was used because larger numbers of charges are provided per mole than with, say, NaCl]. The rates measured were, at 19°: 'salt-free', 6, and, with salt, 5×10^7 M^{-1} sec.$^{-1}$.

Effect of high concentrations of sucrose. In view of the high rate of the combination reaction, experiments in 40 % (w/v) and 60 % (w/v) sucrose were carried out. The reaction appeared to be slightly slowed (about 50 %) by 40 % sucrose and substantially slowed (to one-fifth to one-tenth) by 60 % sucrose in experiments at 18°, with carboxyhaem and globin concentrations of 1 μM after mixing. As it seemed possible that this effect was due to difficulties in mixing the viscous solutions, a control experiment was carried out in which whale myoglobin was allowed to combine with carbon monoxide. Both the myoglobin and the carbon monoxide were dissolved in 60 % (w/v) sucrose which had been prepared in 0·1 M-phosphate buffer, pH 7·1. The reaction was first followed in the stopped-flow apparatus at 440 mμ, and then equal volumes of the two reagent solutions were intimately mixed and transferred to an observation cell suitable for the flash apparatus of Gibson (1959), and the reaction was followed photochemically as described by Gibson (1956), with light of mean wavelength 440 mμ. The total changes of extinction and the rate of the combination reaction agreed within 10 % by the two methods. The concentration of carbon monoxide was so chosen that the rate expressed as a first-

order constant in the control experiment was 50 sec.$^{-1}$, whereas in the carboxyhaem + globin experiments the observed rate in sucrose was only 4 sec.$^{-1}$. It seems therefore that the effect of sucrose is not due to deficiencies in the stopped-flow apparatus.

Effect of alteration of globin. The effect of alkaline denaturation of the globin was examined in experiments in which a portion of globin solution (0·1 mM), after standing for 10 min. at 18° in the presence of 0·1 N-NaOH, was mixed with 50 vol. of 0·1 M-buffer, pH 10·6, and oxygen was removed with dithionite (0·5 mg./ml.). The reaction with carboxyhaem was found to be even more rapid than that of native globin, the observed rate constant at 18° being $1{\cdot}6 \times 10^8$ M^{-1} sec.$^{-1}$. The occurrence of denaturation was checked by spectroscopic observation of the band at 557 mμ formed on reaction of the globin solution with free haem. In view of the very high rates found in all the experiments with carboxyhaem so far described, its reaction with pyridine was next examined. In one experiment in which 3 μM-carboxyhaem was allowed to react at pH 7·1 and 18° with 2·5 mM-pyridine, the rate constant found was 330 M^{-1} sec.$^{-1}$. The reaction is pH-sensitive, but was not investigated in detail. Imidazol was similar to pyridine.

Reaction of mesocarboxyhaem with globin. In a single experiment at pH 9·1 and 20°, of the type shown in Fig. 4, and covering a similar range of concentrations, the observed rate of reaction was found to be $6{\cdot}5 \times 10^7$ M^{-1} sec.$^{-1}$. The experiment was carried out exactly as for protohaem except that observations were made at 409 mμ, the wavelength of maximum absorption of carboxymesohaemoglobin.

Reaction of myoglobin globin with carboxyprotohaem. This protein has been less fully studied than haemoglobin globin, but in three experiments it has been found that the mean rate of combination is $2{\cdot}8 \times 10^7$ M^{-1} sec.$^{-1}$ at pH 9·1 and 20°. The temperature coefficient is 2·0 per 10° temperature rise (one experiment), corresponding, as with haemoglobin globin, to an activation energy of about 10 kcal.

Mechanism of the reaction between carboxyhaem and globin

Anomalous observations. Although the course of the reaction appeared to be satisfactorily second order with very low concentrations of carboxy haem and globin, closer analysis of the data already given shows important inconsistencies. For example, the time course of the reactions illustrated in Fig. 4 differs as between the higher and lower concentrations. In Fig. 6 the reciprocals of the concentrations of carboxyhaem and globin (which were present in equal amounts in this experiment) are plotted against time. If the reaction were accurately second order, a series of parallel straight lines should be obtained. At the higher concentrations there is a marked curvature at the lower ends of the lines, and the slope of the lowest line is distinctly less than that of the others. Further, the rapid changes in extinction observed in the flow apparatus do not increase in proportion with the concentrations of carboxyhaem and globin. In Table 1, the observed and expected changes at 421 mμ are set out, due allowance being made for the dead time of the apparatus (3 msec.). The last column shows that there is a progressive fall in the ratio of the observed to expected change in extinction as the concentration of the reagents is increased.

Two other findings require explanation. The experiments shown in Fig. 6 lead to a velocity constant of about 8×10^7 M^{-1} sec.$^{-1}$, whereas the temperature coefficient derived from Fig. 5 gives an activation energy of about 11 kcal. The corresponding value of the frequency factor is 10^{16}, or about 10^6 times greater than that usually met with in bimolecular reactions. Again, increasing the viscosity 60-fold with sucrose gave a significant

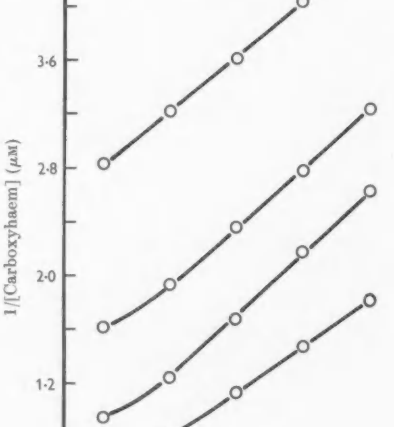

Fig. 6. Treatment of data from Fig. 4 to show deviation from the second-order relation. The ordinate gives the reciprocal of the concentration of carboxyhaem remaining. If the reaction were accurately second order a family of parallel straight lines would be obtained.

Table 1. *Comparison of observed, corrected and calculated changes of extinction*

Extinction changes were (i) observed in flow apparatus from 3 msec. to 5 sec., (ii) corrected for dead time and (iii) calculated from known concentrations of reagents used. Data refer to the experiment of Fig. 4. Figures under 'Corrected' were derived by assuming that the dead time of the flow apparatus is 3 msec. Multiplication of this time by the first-order velocity constant for the reaction being observed gives the natural logarithm of the ratio $\frac{E \text{ at time } 0}{E \text{ at time } 0{\cdot}003}$. 'Calculated' figures suppose a difference in millimolar extinction coefficients at 421 mμ for carboxyhaem and carboxyhaemoglobin of 120, and are obtained by multiplying this figure by the light-path length (2 cm.) and the concentration of reagents given in column 1.

Globin (μM)	E			Corrected/ Calculated
	Observed	Corrected	Calculated	
3·2	0·413	0·505	0·768	0·658
1·64	0·250	0·293	0·394	0·744
0·84	0·149	0·170	0·202	0·842
0·43	0·085	0·094	0·103	0·913

decrease in the rate of reaction, which would not be expected for a reaction with so high an activation energy. Further, the use of sucrose much increased the change in extinction that could be observed.

Suggested mechanism. It appears that the finding set out in the preceding paragraph can be explained by supposing that the reaction takes place in two stages. In the first of these a complex is formed reversibly between carboxyhaem and globin, whereas in the second the carboxyhaem is bound firmly and irreversibly to the globin. The spectroscopic change corresponding to the conversion of carboxyhaem into carboxyhaemoglobin is assumed to take place when the complex is formed.

These suppositions can be formulated as follows (writing y = [globin] = [carboxyhaem]; x = [complex]; k_1 = rate of formation of complex; k_2 = rate of breakdown of complex to carboxyhaem and globin; k_3 = rate of formation of carboxyhaemoglobin from complex):

$$\frac{dy}{dt} = k_2x - k_1y^2, \tag{1}$$

$$\frac{dx}{dt} = k_1y^2 - (k_2+k_3)\,x. \tag{2}$$

These equations do not have an analytical solution. Numerical values have been obtained by standard desk methods and, on a larger scale, with the help of an electronic computer. There is a close analogy between this mechanism and the ordinary Michaelis–Menten formulation of enzyme action, and some of the standard procedures of enzyme kinetics may be applied to give approximate values of the constants in the equations. If it is assumed that k_1 and k_2 exceed k_3 sufficiently, a plot of $1/v$ against $1/S$ may be used to evaluate k_3. In this case, the rate of disappearance of substrate (change in y) is measured, rather than the rate of formation of products, and v is defined as the rate constant for the change in extinction. Adding (1) and (2):

$$\frac{dx}{dt} + \frac{dy}{dt} = -k_3x. \tag{3}$$

On the assumption of equilibrium (i.e. $k_1+k_2 \gg k_3$),

$$k_1y^2 = k_2x, \tag{4}$$

differentiating (4)

$$\frac{2k_1}{k_2}\,y\,\frac{dy}{dx} = 1, \tag{5}$$

substituting in (3)

$$\frac{dy}{dt} = -\frac{k_3y^2}{2y + k_2/k_1}. \tag{6}$$

The pseudo-first-order velocity constant actually measured $= v = -1/y(dy/dt)$ so

$$1/v = \frac{2 + k_2/k_1y}{k_3}. \tag{7}$$

The intercept on the y axis, when $1/v$ is plotted against $1/y$, is thus equal to $2/k_3$, and the slope gives the value of the 'equilibrium' constant k_2/k_1. The progress curve of the reaction may be calculated with the integrated form of (6):

$$k_3t = 2\ln(y_0/y_t) + k_2\frac{(1/y_t) - (1/y_0)}{k_1}, \tag{8}$$

where y_0 and y_t are the amounts of carboxyhaem or globin present at times 0 and t respectively.

Comparison between theory and observation. In Fig. 7 a plot of $1/v$ against $1/y$ shows that a reasonably straight line is obtained for the data of Fig. 4, v being measured in sec.$^{-1}$ over the interval 3–8 msec. after mixing, and y expressed as the mean concentration of free globin during this interval. The progress curves shown as broken lines in Fig. 4 were calculated from the approximate equation (8). These reproduce both the rates and changes of extinction of the experiment with good agreement.

In Fig. 4 the broken lines lie in every case to the left of the point observed 3 msec. after mixing. This is because (8) does not take into account the time required for the formation of the carboxyhaem–globin complex. This time delay offers a rough means of determining the sum of k_1 and k_2. In the example of Fig. 4 it is about 0·8 msec. By numerical computation with (1) and (2), and taking the ratio $k_2/k_1 = 3{\cdot}8$, it is found that, with

$$k_1 = 4{\cdot}75 \times 10^8\ \text{M}^{-1}\ \text{sec.}^{-1},\ k_2 = 1750\ \text{sec.}^{-1}$$

and $k_3 = 375$ sec.$^{-1}$, a good fit to the family of curves of Fig. 4 is obtained. These results are shown as continuous lines in Fig. 4.

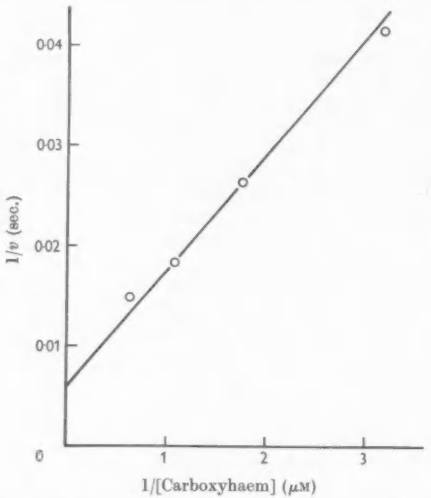

Fig. 7. Relation between the reciprocal of the observed pseudo-first-order velocity constant measured over the time interval 3–8 msec. after mixing and the reciprocal of the concentration of carboxyhaem remaining 3 msec. after mixing. This figure illustrates the relation of equation (7), and leads to $k_2/k_1 \simeq 0{\cdot}27\ \mu\text{M}^{-1}$, $k_3 \simeq 320$ sec.$^{-1}$. For further explanation see text. Data and conditions are from Fig. 4.

Table 2. *Comparison between observed and calculated results for the combination of carboxyhaem and globin*

Concentration of carboxyhaem was 3 μM throughout after mixing borate, pH 9·1, at 18°, 421 mμ, light path 2·0 cm. Figures under give the change at 421 mμ between 3 msec. and 10 sec. after mixing. Rate is the pseudo-first-order velocity constant calculated over the time interval 3–8 msec. after mixing. Calculated values were obtained with $k_1 = 4 \times 10^8\ \text{M}^{-1}\ \text{sec.}^{-1}$, $k_2 = 8 \times 10^2\ \text{sec.}^{-1}$, $k_3 = 2 \times 10^2\ \text{sec.}^{-1}$.

Globin (μM)	*E*		Rate (sec.$^{-1}$)	
	Observed	Calculated	Observed	Calculated
19·5	0·098	0·047	180	177
10·0	0·147	0·102	157	150
5·1	0·223	0·221	102	98
2·6	0·299	0·290	62	61
1·4	0·259	0·130	41	87

A much more stringent test of the assumptions used in these calculations can be obtained by varying the concentration of globin while keeping that of carboxyhaem constant. A part of such an experiment is reproduced in Table 2, which shows the observed and calculated changes of extinction from 3 msec to 10 sec. after mixing, and the observed and calculated rate constants for the change of extinction between 3 and 8 msec. after mixing. The calculated results were obtained by the numerical procedure, with the values given in the table. The results with 2·6 and 5·1 μM-globin agree well: with 10 and 19·5 μM, although the rate constants are in good agreement, the observed changes of extinction are greater than expected. This may be due to the neglect of the mixing time of the flow apparatus, the calculations assuming that complete mixing takes place instantaneously, but is much more likely to be due to the carboxyhaem–globin complex having a lower extinction at 421 mμ than carboxyhaemoglobin. With the lowest globin concentration the change of extinction observed is again greater than expected, whereas the rate constant is only about half the calculated value. This result would be observed if, in addition to combining with the specific sites on the globin to give carboxyhaemoglobin, further carboxyhaem molecules could form a carbon monoxide haemochromogen by combination at non-specific sites on the protein. Such a reaction would, of course, only be observed under conditions where the carboxyhaem was in excess, as the specific sites combine so rapidly with carboxyhaem. In principle, this secondary combination could be examined kinetically and its effects allowed for in calculations of the kind shown in Table 2, if this seemed desirable.

Values of kinetic constants under various conditions

Analyses similar to those just described in detail have been made for experiments under various conditions, and approximate values for the constants are suggested in Table 3. The fit to the experimental data was usually as good as that in Fig. 4, and in all cases the determinations were made with equal concentrations of carboxyhaem and globin.

The results show that both the formation of the carboxyhaem–globin complex and its conversion into carboxyhaemoglobin are influenced strongly by pH and temperature, the stability of the complex being increased tenfold by changing the pH

Table 3. *Results of kinetic analysis of the reaction between globin and carboxyhaem under various conditions*

Observations were made with equal concentrations of carboxyhaem and globin in all cases. The concentrations are those after mixing. Light of wavelength 421 mμ, path 2 cm., was used throughout. Further description of the experiments is given in the text.

Concn. (μM)	Observed rate (sec.$^{-1}$)	pH	Temp.	k_2/k_1 (μM)	k_3 (sec.$^{-1}$)	Special conditions
1·0	11·5	7·1	2°	0·50	23	—
1·0	26	7·1	19	0·36	73	—
1·0	45	7·1	25	0·22	120	—
1·2	46	7	19	0·26	123	Low salt expt.
1·2	30	7	19	0·48	86	Control for low salt expt.
0·44	2·5	7·1	18	10	50	60% Sucrose
0·86	5·2	7·1	18	6	58	60% Sucrose
1·68	8·4	7·1	18	8	57	60% Sucrose
*	*	9·1	19	3·8	375	Values from Fig. 4
1·4	30	9·1	20	1·4	110	Myoglobin globin

* No concentrations or rates are given for the experiment from Fig. 4, as the numbers quoted for the constants are averages from the four individual runs of that experiment.

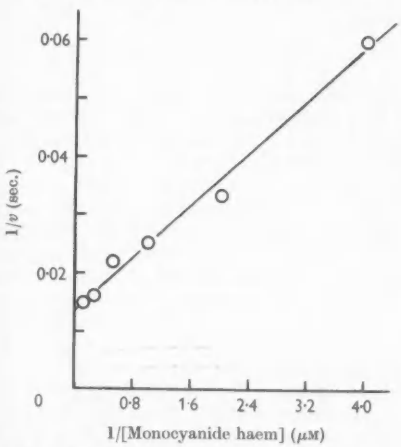

Fig. 8. Combination of monocyanide haem with haemoglobin globin. The plot shows the reciprocal of the pseudo-first-order velocity constant calculated over the period 3–8 msec. after mixing against the reciprocal of the concentration of monocyanide haem present immediately after mixing. Observations were made at 435 mμ, light-path length 2 cm.; borate buffer, pH 9·1; 20°. Values were estimated as described in the text: $k_2/k_1 = 0{\cdot}88\,\mu M^{-1}$, $k_3 = 90$ sec.$^{-1}$.

from 9·1 to 7·1, and k_3 is decreased threefold. The effect of these two factors acting in opposition is to give only a small effect of pH on the observed reaction. The effect of temperature on k_3 allows the activation energy for the step controlled by it to be calculated as 12 kcal.: the ratio k_1/k_2 shows Q_{10} 1·4.

The experiments with 60% sucrose suggest that the increase in viscosity affects primarily the ratio k_2/k_1, which is increased some 20-fold, whereas there is little, if any, effect on k_3. The overall reaction is slowed to about one-fifth, and the proportion of the reaction which can be observed is much increased.

A single set of figures, taken from a more extensive experiment, shows that myoglobin globin and carboxyhaem combine in much the same way as haemoglobin globin.

Combination of monocyanide haem with globin

Although the kinetic analysis of the reactions of carboxyhaem accounts successfully for most of the results, other mechanisms may be proposed to explain them. In particular, a reasonable fit can be obtained with equations (1) and (2) on the assumption that $k_2 = 0$. This can be tested with monocyanide haem, where two kinds of experiment are possible. In one, monocyanide haem is allowed to combine directly with globin; in the other cyanide is displaced from dicyanide haem by globin to give cyanide haemoglobin.

A direct combination experiment is illustrated in Fig. 8. The analysis differs in detail from that of the carboxyhaem experiments: it is found that the observed extinction changes do not decrease strikingly as the concentration of haem and globin is increased (compare Table 1 for carboxyhaem), which suggests that the light absorption of the monocyanide haem–globin complex is similar to that of monocyanide haem at 435 mμ, the wavelength at which the observations were made. The abscissa in Fig. 8 corresponds to $1/(x+y)$, with symbols as defined in equations (1) and (2), instead of $1/y$, and v becomes

$$-\frac{1}{(x+y)}\frac{\mathrm{d}(x+y)}{\mathrm{d}t}$$

in place of

$$-\frac{1}{y}\frac{\mathrm{d}y}{\mathrm{d}t}.$$

The expression analogous to (7) is then

$$\frac{1}{v} = \frac{1}{k_3}\left(1+\frac{\sqrt{[aK+\frac{1}{4}K^2]}-\frac{1}{2}K}{a+\frac{1}{2}K-\sqrt{[aK+\frac{1}{4}K^2]}}\right),$$

where $K = k_2/k_1$ and $a = x+y$. The intercept when $1/a = 0$ is therefore $1/k_3$ instead of $2/k_3$ as in Fig. 7. The reciprocal plot is no longer strictly linear, and bends down rather sharply in the area covered by the two lowest points in the Figure. Values of k_3 and k_2/k_1 have been obtained algebraically from the data of Fig. 8, and are:

$$k_3 = 90 \text{ sec.}^{-1}, \quad k_2/k_1 = 0{\cdot}88\ \mu\text{M}^{-1}.$$

A further special difficulty requires mention: as Keilin (1949) has shown, it is not possible to separate the two steps of cyanide addition to haem completely, even at alkaline pH, so that the concentration of monocyanide haem cannot be regarded as equal to the total haem concentration. In practice, the solutions used were prepared by adding dilute (0·1 %) KCN solution to a solution of haem in borate buffer, pH 9·1, until the sharp α-band of monocyanide haem in the green appeared at its strongest as judged with a pocket spectroscope. The amount of monocyanide haem was then measured approximately by making a spectrophotometric reading at 552 mμ, the maximum of the α-band, and using Keilin's extinction coefficient.

Displacement experiments with dicyanide haem and globin

According to Keilin (1949) haem forms two compounds with cyanide which may be written CN—**H**—H_2O and CN—**H**—CN, where **H** represents the haem nucleus. If the dicyanide compound is unable to combine directly with globin, the reaction must proceed through the intermediate formation of monocyanide haem. This monocyanide haem will either recombine with cyanide to reform dicyanide haem or combine with globin to form the postulated monocyanide haem–globin complex. The complex, in its turn, will either form the relatively stable cyanide haemoglobin, or dissociate to re-form monocyanide haem and free globin. Writing dicyanide haem as y, monocyanide haem as z and monocyanide haem–globin complex as x, the reactions are

$$y \underset{k_5}{\overset{k_4}{\rightleftharpoons}} z + \text{CN}^- : \quad z + G \underset{k_2}{\overset{k_1}{\rightleftharpoons}} x \xrightarrow{k_3} \text{cyanide haemoglobin},$$

where G is the concentration of globin. To examine this scheme experimentally the conditions were adjusted to allow the concentrations of CN^- and globin to be regarded as constant, at least in the early stages of the reaction: further, with high concentrations of cyanide and globin the concentration of monocyanide haem may be regarded as small throughout the reaction and therefore dz/dt can be taken as zero. Then

$$dz/dt = k_4 y + k_2 x - k_5 z\,[\text{CN}^-] - k_1 z\,[G] = 0, \quad (9)$$

$$z = (k_4 y + k_2 x)/(k_5[\text{CN}^-] + k_1\,[G]), \quad (10)$$

$$dy/dt = k_5[\text{CN}^-]\,z - k_4 y, \quad (11)$$

$$dx/dt = k_1[G]\,z - (k_2 + k_3)\,x. \quad (12)$$

On substituting from (10) in (11) and (12) the course of the displacement reaction can be calculated by numerical methods. For the special case $k_2 = 0$, standard methods give the solution

cyanide haemoglobin =

$$y_0\,(Ae^{-k_3 t} - k_3 e^{-At} + k_3 - A)/k_3 - A, \quad (13)$$

where y_0 is the initial concentration of dicyanide haem and

$$A = k_1 k_4\,[G]/(k_1[G] + k_5\,[\text{CN}^-]).$$

To compare theory and experiment, determinations of the rate of the displacement reaction have been made by mixing dicyanide haem in equilibrium with various concentrations of cyanide with globin. This reaction is not easy to follow because the spectrum of dicyanide haem is rather similar to that of cyanide haemoglobin. The results are shown in Fig. 9, where the reciprocal of the rate of replacement is plotted against cyanide concentration. The rate of the reaction is seen to depend on the cyanide concentration, a finding which would not be expected if dicyanide haem were able to react directly with globin.

The quantitative comparison of (11) and (12) with experiment requires that all the velocity constants be evaluated. The ratio k_2/k_1 is already available from the experiments of Fig. 8, together with k_3. The observed rate of reaction with the lowest globin concentration sets a minimum value to k_1, which is taken to be $5{\cdot}6 \times 10^8\ \text{M}^{-1}\ \text{sec.}^{-1}$. From this, k_2 is calculated to be 640 sec.^{-1}. Although these values are only approximate, the rate of the replacement reaction is more sensitive to their ratio than to their absolute values, especially when k_1 exceeds 10^8.

The rate of combination of cyanide with monocyanide haem, k_5, was measured by flash photolysis. It has been found (Q. H. Gibson, unpublished work) that the quantum yield for the conversion of dicyanide haem into monocyanide haem and CN^- ion is high and may be 1, whereas that for the dissociation of monocyanide haem is less than 10^{-4} and may be zero. If therefore a solution of dicyanide haem is flashed, monocyanide haem is formed, which recombines with CN^- ion, giving the

required rate. The velocity has been measured over a range of cyanide concentrations with the results shown in Fig. 10.

The rate of dissociation of dicyanide haem, k_4, has been measured in two ways: (*a*) from the displacement reaction with globin shown in Fig. 9; (*b*) from the reaction with carbon monoxide yielding the compound CN—H—CO (Keilin, 1949). (*a*) For the reaction with globin, which is irreversible, the situation is exactly analogous to the replacement of oxygen in combination with myoglobin by carbon monoxide. The theory of this method has been fully developed by Gibson & Roughton (1955), who have shown that if the reciprocal of the rate of replacement is plotted against the partial pressure of oxygen in the solution, a straight line results whose intercept is equal to the reciprocal of the velocity constant of the reaction oxymyoglobin → myoglobin + O_2. In the present case, if the reciprocal of the rate of displacement of cyanide is plotted against the concentration of cyanide the value of k_4 is obtained. Such a plot is given in Fig. 9.

Exactly similar reasoning may be applied to method (*b*), where the reaction is

$$\text{CN—H—CH} + \text{CO} \underset{k_5}{\overset{k_4}{\rightleftharpoons}} \text{CN—H—CO} + \text{CN}.$$

Since this reaction is reversible, and, at the higher cyanide concentrations, is far from complete, the observed rate constants must be corrected to take into account the total amount of exchange occurring. The details of an experiment are given in Table 4 and the reciprocal plot derived from it is given in Fig. 11. The two methods give results in satisfactory agreement with a mean value for k_4 of 22 sec.$^{-1}$.

Comparison of theory and experiment

The course of the replacement reaction has been calculated from (11) and (12), with the constants quoted, and the results are inserted in Fig. 9. In view of the number of separate experimental determinations involved the agreement is highly satisfactory and strongly supports the suggested mechanism. The time course of the reaction is also interesting, and one example showing the concentrations of dicyanide haem, monocyanide haem–globin complex and cyanide haemoglobin is given in Fig. 12. There is a lag period in the appearance of cyanide haemoglobin, during which the concentration of complex is building up, and the rate

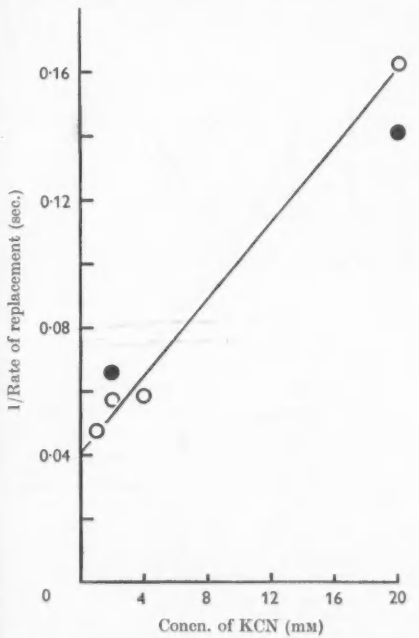

Fig. 9. Formation of cyanide haemoglobin from mixtures of dicyanide haem and haemoglobin globin reacting in the presence of various concentrations of cyanide. The ordinate gives the reciprocal of the observed velocity constant measured over the interval 3–38 msec. after mixing (units, seconds). Initial concentrations: dicyanide haem, 6 μM; haemoglobin globin, 10 μM, dissolved in borate buffer, pH 9·1. Observations were made at 440 mμ, light-path length 2 cm. ○, Experimental observations; ●, results of calculations described in the text.

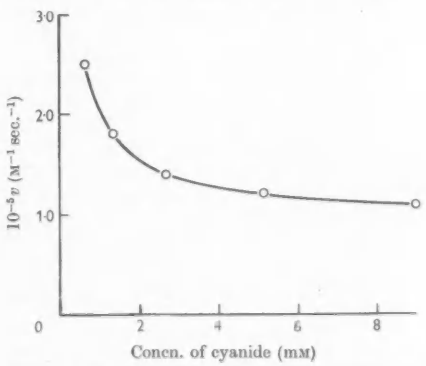

Fig. 10. Combination of cyanide with monocyanide haem. The figure shows the relation between the second-order rate constant for the combination reaction, as determined by flash photolysis, and the concentration of cyanide used in the experiments. Total haem concentration 5 μM; light-path length 1 cm.; observations were made at 435 mμ; temperature 20°; flash energy 200 J.

constant rises over successive time intervals. The experimental observations show a similar acceleration, which is regarded as offering strong confirmation of the correctness of the mechanism proposed. Finally, equation (13) may be used to give a lower limit for k_1 by considering the position where $k_2 = 0$. In order to reproduce the rates of Fig. 9 with $k_2 = 0$, it is necessary to set $k_1 = 4 \times 10^7$. This is too low for the data of Fig. 8, where, at the lowest concentration of monocyanide haem and globin, a value for $k_1 = 1{\cdot}8 \times 10^8$ would be required, with $k_2 = 0$. On returning to the data of Fig. 9 with $k_1 = 1{\cdot}8 \times 10^8$, a value of $k_2 = 200$ sec.$^{-1}$ is required. These values will now not fit the data of Fig. 8, as introduction of a finite value for k_2 slows down the combination reaction. In this way it is found that the values for k_1 and k_2 of $5{\cdot}6 \times 10^8$ and 640 sec.$^{-1}$ are minimum values for the combination reaction.

DISCUSSION

The experiments with haematin and globin are interesting chiefly because the reaction is considerably faster than that observed by Theorell & Maehly (1950) for apoperoxidase and haematins. In spite of the large differences in rate found in individual experiments, the difference between apoperoxidase and haemoglobin is probably significant.

The experiments with carboxyhaem are technically more satisfactory and have allowed a kinetic analysis which accounts for many of the observed features of the reaction: in particular, the combination of a high rate with a high temperature coefficient, as well as the effect of high viscosity. The formation of a complex between the carboxyhaem and globin speeds up the reaction by allowing the final formation of carboxyhaemoglobin to take place whenever the complex acquires sufficient energy to react, instead of requiring a collision to occur at a time when the reactants happen to possess the required energy. In a sense, then, the system may be regarded as a model of enzyme action, illustrating that a reaction may be speeded without a change in activation energy. The point is further emphasized when the results for globin are compared with those

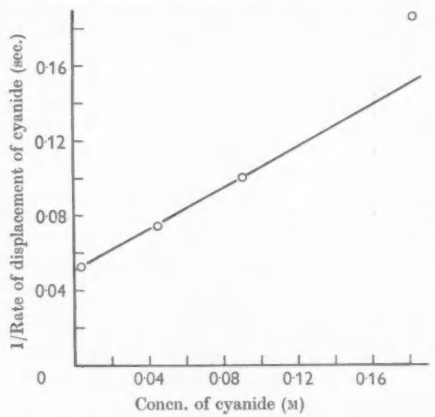

Fig. 11. Displacement of cyanide from dicyanide haem by carbon monoxide. This figure represents the data of Table 4 and leads to a value of 20 sec.$^{-1}$ for the rate constant of the replacement reaction: dicyanide haem + CO→ monocyanide carboxyhaem + cyanide. For further details see text and the legend of Table 4. The deviation of the highest point is due to the large correction required because of the back-reaction at this cyanide concentration.

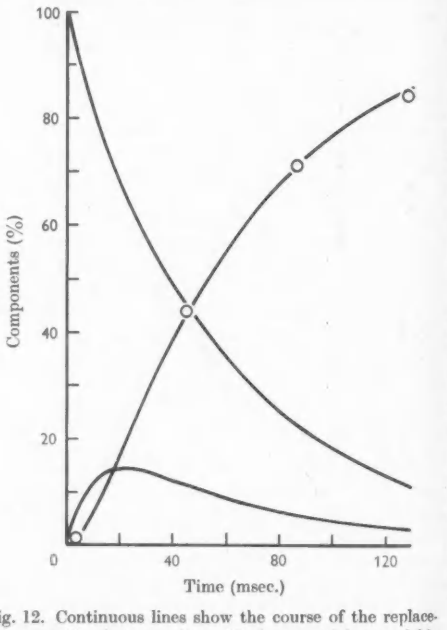

Fig. 12. Continuous lines show the course of the replacement reaction between dicyanide haem and haemoglobin globin as calculated from equations (11) and (12). Ordinate shows: for the descending curve, the percentage of dicyanide haem remaining; for the ascending curve, the percentage of cyanide haemoglobin that has been formed; for the lowest curve rising to a maximum, the percentage of monocyanide haem–globin complex. Points represent a progress curve of the reaction as observed experimentally under the conditions specified in the legend to Fig. 9 with the time scale altered to show the agreement in form. Values used in obtaining the calculated curve: $k_1 = 5{\cdot}6 \times 10^8$ M^{-1} sec.$^{-1}$, $k_2 = 640$ sec.$^{-1}$, $k_3 = 90$ sec.$^{-1}$, $k_4 = 20$ sec.$^{-1}$, $k_5 = 2 \times 10^5$M^{-1} sec.$^{-1}$. Cyanide concentration, 1 mM; globin concentration, 10 μM.

for pyridine and imidazol where the second-order constants are of the order of 10^2 M^{-1} sec.$^{-1}$ in both cases: with solutions as dilute as those used in the experiments with globin the rate of combination would have been decreased to about one-millionth.

The rate of the first step in the reaction, corresponding to k_1 in the scheme, is not known with assurance; the values used must be regarded as minimum ones. For carboxyhaem this statement rests on the finding that equilibrium between the complex and the haem and globin is virtually complete in less than 3 msec., the dead time of the stopped-flow apparatus. The experiments with monocyanide haem support a similar value for the initial combination rate of this compound: in this case additional evidence is available from the correlation of the rates of the direct combination reaction with the rate of displacement of cyanide from dicyanide haem by globin. Thus in both cases it seems necessary to assume a rate of about 5×10^8 M^{-1} sec.$^{-1}$ for the initial step. Although high, such a value is quite possible in view of the investigation by Alberty & Hammes (1958) of the rate at which a diffusion-controlled reaction can take place with a specific site freely accessible from all points in a hemisphere. The effect of decreasing the salt concentration on the ratio k_2/k_1 (Table 3) is also in the direction to be expected from the later investigation of Hammes & Alberty (1959) of electrostatic effects in such reactions. Similarly, the effect of 60% sucrose on the reaction may be explained by supposing that k_1 is much reduced by the increase in viscosity. It is likely that k_2 would also be decreased because, in the viscous solution, a molecule of carboxyhaem which had dissociated from the globin would have a lessened chance of diffusing out of the reaction radius, and so would tend to recombine almost at once with the reaction site. The effect of pH may be discussed in electrostatic terms: as at the most acid pH examined (pH 7·1) the carboxyl groups of the haem will still be almost completely ionized, the effect would be due entirely to change in the ionization of groups in the protein near to the specific site. Unfortunately the range of pH which can be used is small because neither the carboxyhaem nor the globin solution is stable over a wide range.

The specificity of the combination reaction is not high since both myoglobin globin and denatured globin react at about the same rate as native globin: further, the substitution of mesohaem for protohaem does not greatly affect the results. No experiments with other proteins have been made, but since Kiese & Kurz (1958) have found that a concentration of 0·03 mM free protohaem is required for half-saturation of plasma albumin it is evident that globin has an affinity of an altogether higher order.

The work of Rossi-Fanelli *et al.* (1958) has shown that at neutral pH and high ionic strengths (between 0·1 and 0·3) native human globin has an average molecular weight of 41 000 and is thought to consist mainly of molecules with half the molecular weight of haemoglobin. The stability to changes in pH and temperature are also quite different from those of haemoglobin. Rossi-Fanelli *et al.* (1958) have shown that all these differences between globin and haemoglobin disappear after the reconstitution reaction. Since most of the globin molecules are half-sized it is likely that we have been dealing in this paper only with a first step in the reconstitution of haemoglobin and that a complete description of the reaction must include an account of the process by which the molecular weight returns to 68 000. It is intended to follow the later stages of the reaction by a variety of methods, including light-scattering, and rapid thermal methods as well as more conventional kinetic procedures.

The results with monocyanide haem, which has one more negative charge than carboxyhaem, do not show wide differences from those with carboxyhaem, but as the experiments were all carried out in solutions of high ionic strength, effects due to charge would be minimized. In addition to the difficulties in preparing solutions of monocyanide haem of known concentration, it was necessary to take into account the instability of cyanide haemoglobin, described by Keilin & Hartree (1955). In confirmation of their work it was found that in the course of a minute or so the spectrum of cyanide haemoglobin was replaced by that of reduced haemoglobin. This change was slower with high cyanide concentrations. In no case did the change interfere with our kinetic determinations, which were always complete in less than 1 sec.

A difficulty in the analysis of the results which has not been fully dealt with in the Experimental section is due to lack of knowledge of the absorption spectrum of the intermediate haem–globin complexes. It has been assumed that at 421 mμ the carboxyhaem–globin complex has the same absorption as carboxyhaemoglobin, but that the monocyanide haem–globin complex has, at 435 mμ, the same absorption as monocyanide haem. It is possible, by detailed examination of the changes of extinction in experiments forming part of a series, to arrive at a rough estimate of the true values. It appears that the errors in k_2/k_1 and k_3 due to the assumptions about the absorption coefficient are less than 10%, but the fit in Fig. 4 could be improved by altering the value taken for the extinction coefficient of the complex.

The experiments with dicyanide haem show that this compound does not react directly with globin. Carboxyhaem does react directly, since the rate of

Table 4. *Displacement of cyanide from dicyanide haem by carbon monoxide*

Dicyanide haem in equilibrium with KCN of the concentration shown in column (1), dissolved in borate buffer, pH 9·1, was mixed with 1 mM-carbon monoxide also dissolved in borate buffer. The extinction change recorded in column (2) was observed at 434 mμ with a path length of 2·38 mm. The 100% value used in calculating column (3) cannot be observed directly: it was obtained by extrapolation of the data of column (2) to zero cyanide concentration. Column (4) gives the rate constant with which the extinction change of column (2) took place. Column (5) gives the rate constant that would have been observed if complete displacement had occurred in each case. Column (6) gives

$$K = [\mathbf{H}\text{-}(CN)_2][CO]/[\mathbf{H}\text{-}(CN)(CO)][KCN],$$

where **H** is the haem nucleus.

Cyanide (M) (1)	ΔE (2)	ΔE (as percentage) (3)	Rate constant (sec.$^{-1}$) (4)	$\frac{\text{Rate constant} \times \Delta E}{100}$ (5)	Equilibrium constant (6)
0·182	0·026	14·8	36·4	5·4	0·030
0·091	0·055	31·4	31·8	10·0	0·024
0·045	0·090	51·4	26·4	13·6	0·022
0·0018	0·168	96·0	20·0	19·2	0·024
100%	0·175	—	—	—	—

reaction is independent of the concentration of carbon monoxide in solution. This result appears to prove that carboxyhaem is monomeric, since, if it were dimeric with the formula CO—**H**—**H**—CO, no reaction could take place with globin until a molecule of carbon monoxide had dissociated from the dimer. This result confirms Smith's (1959) suggestions, based on spectroscopic evidence. The data of Table 4 may be used to calculate the rate of combination of carbon monoxide with monocyanide haem, by the method of Gibson & Roughton (1955). The value found is 1×10^7 M^{-1} sec.$^{-1}$, which compares with the value of $1{\cdot}6 \times 10^7$ M^{-1} sec.$^{-1}$ found by Smith (1959) for the combination of carbon monoxide with free haem.

The experiments throw some light on the accessibility of the haem site to carboxyhaem and monocyanide haem; it seems fair to conclude that in the stage of complex formation the haem molecule lies on or near the surface of the globin. It is not possible to say what chemical groups are involved in complex formation, particularly as the spectrum of the complex in one case resembles carboxyhaemoglobin, and in the other, monocyanide haem. Work with a variety of haems (e.g. without carboxyl groups) may help in this respect.

Recently Perutz *et al.* (1960) have completed the task of describing the polypeptide chains of the haemoglobin molecule. Although this work is a landmark in the history of haemoglobin chemistry it does not seem possible to discuss our results in terms of their findings. Globin and haemoglobin differ widely in many ways, and the polypeptide chains in a solution of globin may be arranged differently from those in the oxyhaemoglobin crystal. It is interesting, however, that the similarity between the chains in myoglobin and haemoglobin revealed by X-ray analysis is duly reflected in similar kinetic behaviour with carboxyhaem and haematins. It can also be supposed that after primary complex formation the secondary process described by k_3 corresponds to a folding of the globin chain around the haem group to give a stable form similar to that described by Perutz *et al.* (1960), but this is no more than a speculation.

SUMMARY

1. Proto-, meso- and deutero-haematin react with human globin at a rate determined by the properties of the haematin.

2. Carboxyprotohaem reacts rapidly with globin. An intermediate reversible complex is formed with combination and dissociation velocity constants of 5×10^7 M^{-1} sec.$^{-1}$ and 1700 sec.$^{-1}$ respectively at pH 9·1 and 19°.

3. The complex is converted into carboxyhaemoglobin in a first-order process with rate constant 370 sec.$^{-1}$ at 19° and pH 9·1. The activation energy is 12 kcal.

4. The effects of pH, viscosity and temperature on the reaction of carboxyhaem with globin have been examined.

5. Similar results have been obtained with monocyanide haem both by following the direct reaction and by observing the rate of replacement of cyanide in dicyanide haemoglobin by globin.

We wish to thank Mr P. H. Blundell and Mrs A. Fairburn of the Department of Applied Mathematics, University of Sheffield for preparing a programme and computing numerical solutions of equations (1) and (2). We are indebted to the Consiglio Nazionale delle Ricerche and the Research Fund of the University of Sheffield for grants in aid of travelling expenses and to the Medical Research Council and the Rockefeller Foundation for providing much of the special equipment needed.

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Biochem. J. (1960) **77**, 341

Intermediates in the Catalytic Action of Lipoyl Dehydrogenase (Diaphorase)

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Lipoyl dehydrogenase was first discovered in bacteria and shown to be involved in the oxidation of keto acids (Gunsalus, 1954; Reed, 1957). A similar involvement has been shown in mammalian tissues, particularly in the pig-heart α-oxoglutarate–dehydrogenase complex (Sanadi, Langley & White, 1959). Massey (1958, 1960*a*) demonstrated that the lipoyl dehydrogenase of pig-heart muscle is a flavoprotein identical with the flavoprotein which was isolated by Straub (1939) and studied by Savage (1957). This conclusion has been confirmed by Searls & Sanadi (1959), but has also been dismissed (Ziegler, Green & Doeg, 1959). A likely explanation of the inability of Ziegler *et al.* to observe lipoyl-dehydrogenase activity in their diaphorase preparation has, however, been found in the sensitivity of this enzyme to trace metals (Veeger & Massey, 1960), which can abolish the activity towards lipoid acid although at the same time it greatly stimulates the activity towards 2:6-dichlorophenol-indophenol, a dye commonly used to assay diaphorase activity. Further studies of this enzyme have shown that two, rather than one, molecules of pyridine nucleotide take part in the reaction:

Lipoic acids + reduced diphosphopyridine nucleotide + H^+ ⇌ reduced lipoic acids + diphosphopyridine nucleotide

and that a protein dithiol group is formed as an intermediate (Massey & Veeger, 1960).

Savage (1957) had reported that on the addition of reduced diphosphopyridine nucleotide to diaphorase there was incomplete bleaching of the 450 mμ band of the flavin and an increase in absorption at wavelengths greater than 500 mμ. This observation has been confirmed by Searls & Sanadi (1959). The formation of a red colour with many flavoproteins on the addition of substrates has been reported: e.g. old yellow enzyme (Haas, 1937); reduced diphosphopyridine nucleotide peroxidase (Dolin, 1956, 1957); acyl-coenzyme A dehydrogenases and snake-venom L-amino acid oxidase (Beinert, 1957); diaphorase (Savage, 1957); dihydro-orotic dehydrogenase (Friedmann & Vennesland, 1958); D-amino acid oxidase (Massey & Palmer, 1960). The cause of such a red colour has been the subject of much discussion. In general, the formation of a red colour has been taken to indicate the existence of the enzyme flavin in a half-reduced (or semiquinone or free radical) form, by analogy with the studies on free flavins (Kuhn & Wagner-Jauregg, 1934; Michaelis, Schubert & Smythe, 1936; Beinert, 1956). With reduced diphosphopyridine nucleotide peroxidase, however, Dolin has presented much evidence that makes it difficult to accept this interpretation of the spectral changes observed with substrates and dithionite. At the moment no generalizations can be made;

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each enzyme must be considered separately as a particular case. In this paper results are presented which suggest that with lipoyl dehydrogenase the red colour produced by substrate is due to the formation of a flavin semiquinone. The role of this form in the catalytic action of the enzyme has been investigated; its formation by reduced lipoic acids and its reoxidation by oxidized lipoic acids are the rate-determining steps in the reaction catalysed by this enzyme. A preliminary account of this work has been presented to the Symposium on Free Radicals in Biological Systems at Stanford, California.

EXPERIMENTAL

Materials

Chemicals. Reduced diphosphopyridine nucleotide (DPNH), diphosphopyridine nucleotide (DPN) and *p*-chloromercuriphenyl sulphonate were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). DL-α-Lipoic acid was a gift from Dr I. C. Gunsalus. DL-Lipoylglycylglycine, dihydrolipoylglycine and dihydrolipoamide were gifts from Dr L. J. Reed. The bovine-serum albumin was the crystalline product of The Armour Laboratories (Eastbourne). Sodium dithionite (not less than 85%) and ethylenediaminetetra-acetate, disodium salt (EDTA), were from British Drug Houses Ltd. Solutions of the latter compound were neutralized with NaOH in making up standard concentrations. All other chemicals used were of analytical-reagent quality. All the experiments described in this paper were performed in the presence of sodium phosphate buffers, at pH 6·3, pH 7·0, pH 7·2 or pH 7·6. Stock 0·3M-buffers of these pH values were prepared by mixing 0·3M-solutions of Na_2HPO_4 and NaH_2PO_4 (22:78 for pH 6·3; 61:39 for pH 7·0; 71:29 for pH 7·2; 86·5:13·5 for pH 7·6, all v/v). All solutions were prepared with glass-distilled water.

Enzyme preparations. Lipoyl dehydrogenase was made by a slight modification of the method described by Massey (1960*a*). The present method gives larger yields of enzyme, chiefly because of an early ethanolic heat-treatment, which appears to dissociate lipoyl dehydrogenase from the α-oxoglutarate–dehydrogenase complex. With the method previously described, in which all purification steps were carried out at 4°, one-third to one-half of the lipoyl dehydrogenase was discarded in the first $(NH_4)_2SO_4$ fractionation (0–0·35 saturation) because it was still associated with the oxoglutarate–dehydrogenase complex (cf. Massey, 1960*b*).

The modifications of the previous preparation were:

(i) The extract of the pig-heart particles made with 1% (w/v) $(NH_4)_2SO_4$ was heated at 45° for 15 min. with continuous stirring in the presence of 3% (v/v) ethanol and 0·06 mM-EDTA. After cooling, the precipitate which had formed was centrifuged off in a Servall refrigerated centrifuge at 10 000 *g* for 20 min. The supernatant was treated as previously described and after fractionation on a calcium phosphate gel–cellulose column had 60–80% of the specific activity of the final preparation of Massey (1960*a*). (ii) The product from the calcium phosphate gel–cellulose column was further purified by heating at 68–70° for 5 min. in the presence of 0·05M-sodium phosphate, pH 7·0, 0·1M-$(NH_4)_2SO_4$ and 0·1 mM-EDTA, protein concentration 2–6 mg./ml. The white precipitate formed was centrifuged off in a refrigerated centrifuge at 25 000 *g* for 10 min. and the yellow supernatant was brought to 0·55 saturation with $(NH_4)_2SO_4$ (34 g./100 ml. of supernatant). Any precipitate which formed when the solution was allowed to stand for 15 min. at 0° was centrifuged off and discarded. The supernatant was brought to 0·7 saturation by adding 10 g. of $(NH_4)_2SO_4$/100 ml. of 0·55-saturated supernatant. After 15 min. at 0° the yellow precipitate of enzyme was centrifuged off and dissolved in a suitable buffer, e.g. 0·03M-phosphate, pH 7·0. The product was identical with the enzyme previously described by Massey (1960*a*), with an overall yield from the first extract of 60–85%.

Methods

Enzyme assays. All assays were performed spectrophotometrically at 340 mμ in the thermostatically controlled cell chamber of a Beckman model DU spectrophotometer. Apart from the experiment reported in Fig. 7, all assays were carried out at 25±0·2°. Optical cells of 1 cm. light path were used throughout and the total volume in each assay was 3 ml. Each cell contained 150 μmoles of phosphate buffer of the appropriate pH value, 3 μmoles of EDTA and 2 mg. of bovine-serum albumin. When studying the oxidation of DPNH by oxidized lipoic acids the buffer used was at pH 6·3; reaction was started by adding successively 0·6–4·0 μmoles of DL-lipoic acid or 0·3–4·0 μmoles of DL-lipoylglycylglycine, 0·15 μmole of DPNH, 0·2 μmole of DPN and an appropriate amount of enzyme (an amount such that the extinction change/min. was less than 0·1; the amounts used in a particular experiment are given in the legends to the Figures in the Results section). When studying the reduction of DPN by reduced lipoic acids the buffer used was at pH 7·6; reaction was begun by the successive addition of 0·15–2·0 μmoles of DPN, 0·15–2·0 μmoles of dihydrolipoylglycine or dihydrolipoamide and an appropriate amount of enzyme (as described above). Details of the amounts of substrates and enzyme are given in the Results section. Solutions of dihydrolipoic acid derivatives were prepared as soon as possible before use; 0·3 mM-EDTA was present in such solutions to prevent metal-catalysed oxidation.

Determination of anaerobic spectra. The influence of various substrates and reagents on the absorption spectrum was studied in specially constructed tubes in which a glass tube of 1 cm. internal diameter, furnished with two projecting side arms and topped by a B14 standard ground-glass socket, was fused to an optical cell of 1 cm. light path (Beckman cat. no. 2097). After placing the enzyme and reagents in the appropriate compartments the cell was closed with a B14 standard ground-glass cone fitted with a stopcock. All ground-glass joints were greased and the tube was evacuated on a water pump, filled with O_2-free N_2, evacuated again and the whole cycle was repeated at least four times, the final step being an evacuation.

Preparation of enzyme for stopped-flow experiments. Purified enzyme (Massey, 1960*a*) was dialysed overnight against 0·05M-phosphate buffer, pH 6·3 or pH 7·6. Before use, dialysis tubing was soaked inside and out with 1 mM-EDTA for at least 10 min. and then washed with glass-distilled water. After dialysis, the enzyme was adjusted to the required concentration (see description of individual experiments) by dilution with the appropriate 0·05M-

buffer. Substrate solutions were prepared by dilution from stock solutions and were made up so that they contained a final concentration of 0·05 M-phosphate buffer. Unless otherwise noted a small amount (0·3 mM) of EDTA was also present in all experiments. When the reoxidation by oxidized lipoic acids was studied, the enzyme semiquinone was prepared anaerobically as described in the previous section (except that the final gas phase was O_2-free N_2) by tipping from the side arm the required amount of DPNH. The partially reduced enzyme was then immediately transferred with a hypodermic syringe to the stopped-flow apparatus.

Stopped-flow determinations. These were carried out with the apparatus of Gibson (1954) modified by the use of a 2 cm. light-path observation tube as described by Gibson & Antonini (1960), and by the use of a Hilger D246 monochromator as a source of light. With this equipment extinction changes are recorded photographically from 3 msec. after mixing of the reagents for as long as is required. The concentrations of the solutions were adjusted so that a change in extinction from 0·01 to 0·06 in the 2 cm. light path was observed.

Expression of results

Enzyme concentration and turnover numbers. It is convenient to distinguish between experiments where the amount of enzyme was small compared with the amounts of substrates, and the stopped-flow experiments, where the amounts of enzyme and substrates were of similar orders. The first are called 'catalytic experiments' or 'experiments with catalytic quantities of enzyme' and the others are called 'stopped-flow experiments'.

The enzyme concentration was calculated assuming that the molar-extinction coefficient of lipoyl dehydrogenase at 455 mμ is the same as that of flavinadenine dinucleotide at 450 mμ, i.e. $1{\cdot}13 \times 10^4$ cm.²/m-mole (Whitby, 1953). $E_{340\,m\mu\ DPNH}$ was taken as $6{\cdot}22 \times 10^3$ cm.²/m-mole (Kornberg & Horecker, 1953).

Turnover numbers from the catalytic experiments are expressed as moles of substrate oxidized or reduced/min./mole of enzyme [as shown by Savage (1957), there is 1 mole of flavinadenine dinucleotide/mole of enzyme]. The extinction changes in stopped-flow experiments were expressed as first-order velocity constants in units of $min.^{-1}$, which are then directly comparable with turnover numbers as defined above.

RESULTS

Effect of substrates on the absorption spectrum. Fig. 1 shows the effect on the visible spectrum of diaphorase produced by the anaerobic addition of either DPNH or dihydrolipoamide. A similar change was produced on the addition of dihydrolipoylglycine. The strong fluorescence of the untreated enzyme disappeared almost completely under these conditions. Fig. 2 shows the effect of the addition of DL-α-lipoic acid after the addition of DPNH. It is evident that the oxidized substrate causes a return of the spectrum almost to that of the oxidized enzyme. The extinction changes at 530 mμ produced by anaerobic titration of the oxidized enzyme with DPNH are plotted in Fig. 3, which shows that a stoicheiometric reaction has occurred with maximal extinction change on the addition of 1 mole of DPNH/mole of enzyme. These results were obtained with anaerobic spectrophotometer cells, and the readings were taken 30 sec.

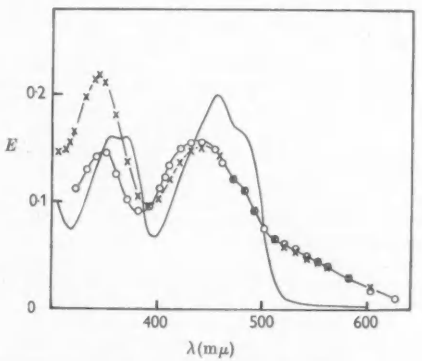

Fig. 1. Effect of DPNH or dihydrolipoamide on the absorption spectrum (1 cm. light path) of pure diaphorase in 0·05 M-phosphate, pH 7·6, at 25°; enzyme concentration 1·2 mg./ml. —, Oxidized enzyme; ×—×, after addition of 1·9 moles of DPNH/mole of enzyme; ○—○, after addition of 18 moles of dihydrolipoamide/mole of enzyme. Additions of reducing substrates were made, and spectra were determined under anaerobic conditions (see Experimental section), in two separate experiments. The total volume in each was 3 ml.; the spectra shown are corrected for the slight volume changes after addition of DPNH or dihydrolipoamide.

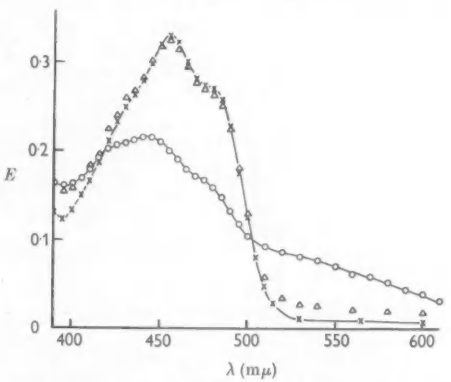

Fig. 2. Effect on the anaerobic absorption spectrum of diaphorase (2·0 mg./ml.) of DPNH followed by lipoic acid. Temperature 25°; 0·05 M-phosphate buffer, pH 6·3. ×, Original oxidized enzyme; ○, after addition of 3·0 moles of DPNH/mole of enzyme; △, after further addition of 40 moles of DL-α-lipoic acid/mole of enzyme.

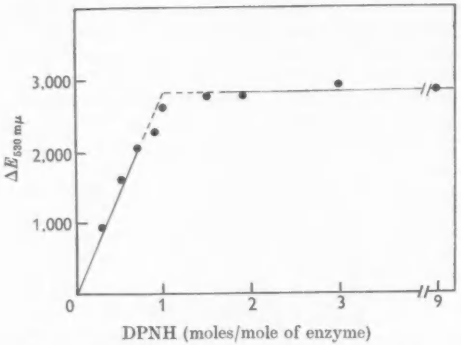

Fig. 3. Increase in $E_{530\ m\mu}$ produced by the anaerobic addition of DPNH to diaphorase in 0·05 M-phosphate, pH 7·6, at 25°. Each point represents the result obtained in a separate experiment, in which DPNH was added to a solution of diaphorase (1·2 mg./ml.). Increase in $E_{530\ m\mu}$ was determined immediately after mixing (30 sec.) with a Beckman DU spectrophotometer. Actual increases in $E_{530\ m\mu}$ ranged from 0·017 with the lowest amount of DPNH to 0·053 with the larger amounts of DPNH. The molar ratio of DPNH to enzyme was calculated assuming that $E_{455\ m\mu}$ of oxidized enzyme was $1{\cdot}13 \times 10^4$ cm.²/m-mole and the $\Delta E_{530\ m\mu}$ was calculated on the same assumption.

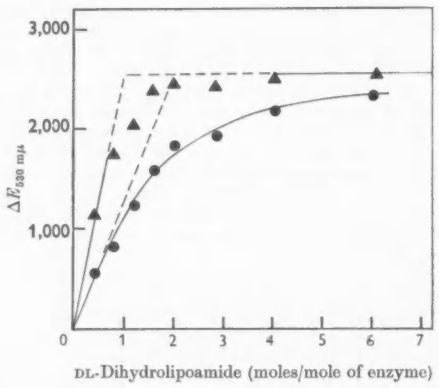

Fig. 4. Increase in $E_{530\ m\mu}$ produced by the addition of DL-dihydrolipoamide to diaphorase in 0·05 M-phosphate, pH 7·6, at 25°. Results were obtained by the stopped-flow method. Final concentration of enzyme in the reaction chamber was 12·3 μM, with the molar ratios of DL-dihydrolipoamide shown. ●, Increase in extinction during the rapid phase of the reaction; ▲, total increase in extinction (rapid + slow phases).

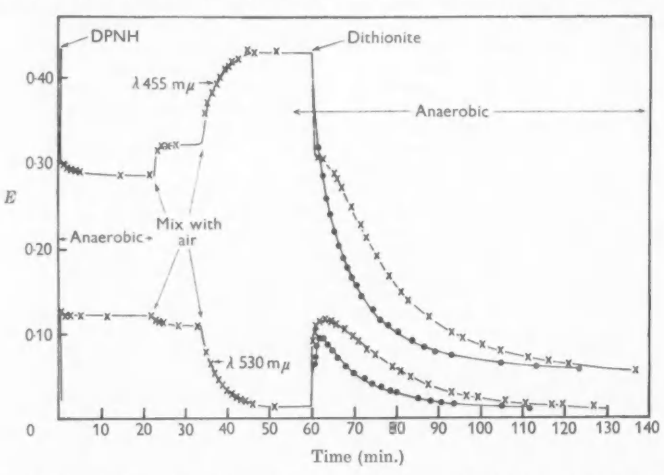

Fig. 5. Effect of DPNH, O_2 and dithionite on $E_{455\ m\mu}$ and $E_{530\ m\mu}$ of diaphorase (2·58 mg./ml.) in 0·05 M-phosphate, pH 7·6, at 25° (total volume 3 ml.). Changes were followed with time in two separate anaerobic cells. In the first experiment (×) 4·4 moles of DPNH/mole of enzyme were added anaerobically. At 22 min. air was admitted and the tube inverted once to bring into contact with air. At 34 min. the tube was inverted once again to mix with air. At 53 min. 10·8 moles of dithionite/mole of enzyme were introduced into a side arm of the cell and anaerobic conditions were re-established. At 60 min. the dithionite was mixed with the enzyme. In the other experiment (●), the same amount of enzyme, which had no previous treatment with DPNH and O_2, was mixed anaerobically with 5·4 moles of dithionite/mole of enzyme. For convenience this reaction is shown to begin at 60 min. In both experiments the dithionite solution (10 mM) was made up freshly with cold de-aerated 0·05 M-phosphate, pH 7·6 (containing 0·6 mM-EDTA) immediately before use.

after mixing. It is shown in a later section, however, that these extinction changes occur within milliseconds after mixing. Fig. 4 shows a similar plot of the extinction increases at 530 mμ, produced by the addition of graded amounts of DL-dihydrolipoamide, measured with the stopped-flow apparatus. On the addition of the compound there is a distinct biphasic increase in absorption, a very rapid reaction (half-time < 20 msec.) followed by a much slower reaction (half-time about 3 sec.). This probably reflects the differences in reaction rates of the natural and unnatural optical forms of the dihydrolipoamide with the enzyme. The extinction changes occurring in the fast phase of the reaction are half those of the total change (fast plus slow) when the total amount of DL-dihydrolipoamide is < 1 mole/mole of enzyme. Above this range the proportion of the rapidly produced change increases relative to that of the slow change; at a molar ratio of DL-dihydrolipoamide:enzyme of 6, 90% of the total change occurs during the rapid phase.

Effect of oxygen and dithionite on the absorption spectrum. Fig. 5 shows the changes in extinction at 455 and 530 mμ in an experiment designed to determine the effect of O_2 and dithionite on the red form of the enzyme produced by DPNH. In this experiment DPNH was added anaerobically and the decrease in extinction at 455 mμ and the increase in extinction at 530 mμ were followed with time. After the immediate changes noted very little further change occurred, and during this time the spectrum was recorded. After 22 min. air was admitted and the contents of the tube were mixed gently once in order to bring them into contact with the air. A small increase in extinction at 455 mμ and a small decrease at 530 mμ occurred; further changes were very slow until the tube was again inverted once after 34 min. A gradual increase in extinction at 455 mμ accompanied by a corresponding decrease at 530 mμ occurred until the absorption spectrum of the original oxidized enzyme was regained. Freshly prepared dithionite was then added to a side arm of the tube and the contents were made anaerobic as described in the Experimental section. At 60 min. the dithionite was tipped into the enzyme+DPN solution (during the oxidation with air the 340 mμ absorption due to the added DPNH disappeared). There was a fairly rapid decrease in extinction at 455 mμ and a corresponding increase at 530 mμ to close to the values originally obtained on the addition of DPNH. This was followed by a slow decrease in both the 455 mμ and the 530 mμ extinction values, extending over a period of 1 hr. under these conditions. Fig. 5 also shows, for comparison, the effect of adding dithionite alone. It is clear that the red colour also appears as an intermediate under these conditions but, whereas the total changes in extinction are the same as when DPNH is present, the rate of change is faster, suggesting that the small molar excess of DPNH over enzyme present may protect the flavin from complete reduction. The spectra recorded at various stages in this experiment are given in Fig. 6. Ferricyanide also causes a rapid return of the spectrum to that of the oxidized form when added to the red form made with DPNH. This oxidation reaction is being studied by the stopped-flow technique; the results will be reported separately.

Effect of p*-chloromercuriphenyl sulphonate on the red intermediate.* When the red intermediate is produced by the addition of DPNH to lipoyl dehydrogenase in an anaerobic experiment, the subsequent addition of the sulphydryl reagent causes a further bleaching of the visible colour, giving a spectrum similar to that of the fully reduced enzyme obtained by the addition of dithionite. The major part of the extinction change (which results in the complete loss of the red colour) occurs within the first 10 min.; this is followed by a further, slower change. The results of such an experiment are shown in Fig. 7. With larger molar ratios of DPNH to enzyme than those used in obtaining Fig. 7 the full spectral changes were completed very rapidly (within 3–5 min.). These experiments must be done at low temperatures, otherwise turbidity develops rapidly. If *p*-chloromercuriphenyl sulphonate is added before DPNH, no reduction of the enzyme flavin occurs, as judged by the lack of changes in extinction either at 455 mμ or 530 mμ. The possible significance of these observations is considered in the Discussion.

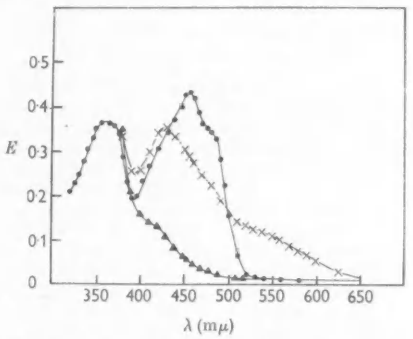

Fig. 6. Spectra recorded at various times in the experiment described in Fig. 5. ●, Oxidized enzyme, before any additions; ×, after addition of DPNH, read between 7 and 21 min.; ▲, after addition of dithionite to ×, read after 130 min.

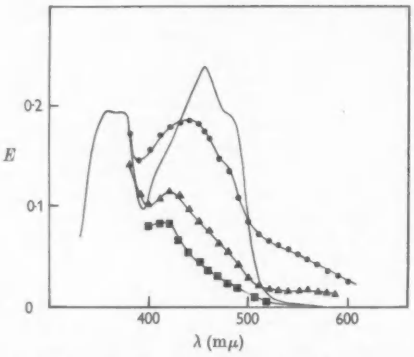

Fig. 7. Effect of *p*-chloromercuriphenyl sulphonate on the absorption spectrum of diaphorase after the addition of DPNH. Enzyme concentration, 1·44 mg./ml. (21·2 μM) in 0·05 M-phosphate, pH 7·2, temperature 5±1°. —, Oxidized enzyme; ●—●, after the addition of 2·9 moles of DPNH/mole of enzyme (spectrum recorded 5–20 min. after addition of DPNH: no significant changes were observed in this time); ▲—▲, after the further addition of 8·2 moles of *p*-chloromercuriphenyl sulphonate/mole of enzyme (spectrum recorded 20–30 min. after the addition); ■—■, same as ▲—▲, but 15 hr. later.

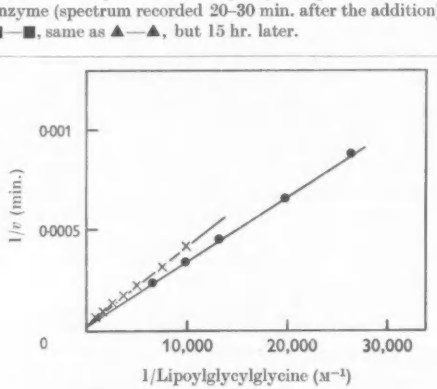

Fig. 8. Effect of DL-lipoylglycylglycine on the rate of oxidation of DPNH by catalytic quantities of enzyme (×), and on the rate of FADH' oxidation (●), measured by the stopped-flow technique. Catalytic rates were calculated as turnover numbers (moles of DPNH oxidized/min./mole of enzyme); the rates of FADH' oxidation were calculated as the first-order rate constants (min.$^{-1}$) for the extinction change at 530 mμ. In both experiments the buffer was 0·05 M-phosphate, pH 6·3; temperature 25°. In the 'catalytic' experiments the conditions were as given in the Experimental section; reaction was begun by the addition of 0·11–0·33 μg. of enzyme. In the stopped-flow experiments FADH' was made anaerobically by the addition of 0·75 mole of DPNH/mole of enzyme and transferred immediately to the stopped-flow apparatus, where it was mixed with the concentrations of lipoylglycylglycine shown. Final concentration of enzyme in the reaction chamber was 15·3 μM.

Rate of formation of the red intermediate by reduced diphosphopyridine nucleotide. When DPNH is mixed with lipoyl dehydrogenase the red colour is produced so rapidly that it is impossible, with the present apparatus, to estimate accurately the rate of formation, except at concentrations of DPNH about the same as those of the enzyme. Thus in stopped-flow experiments at pH 6·3 and 25° when enzyme at a final concentration of 21·5 μM was mixed with a final concentration of 20 μM-DPNH, the first-order rate constant for the increase in extinction at 530 mμ was 8400 min.$^{-1}$. Increasing the concentration of DPNH five-fold resulted in such a rapid formation of the red colour that no quantitative measurement of its rate could be made. It was confirmed, however, that the full extinction change at 530 mμ (expected to follow

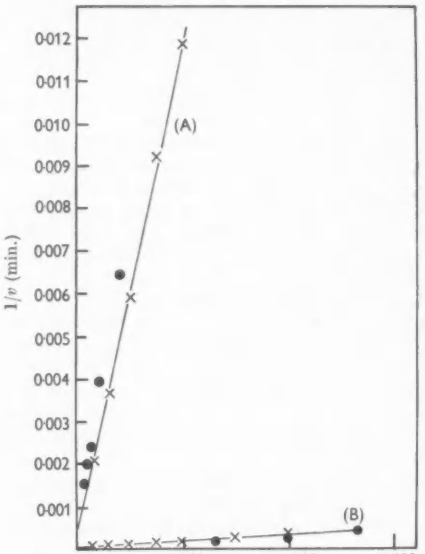

Fig. 9. (A) Effect of DL-lipoic acid on the rate of catalytic oxidation of DPNH (×), and on the rate of FADH' oxidation (●), measured by the stopped-flow method. In both experiments the buffer was 0·05 M-phosphate, pH 6·3; temperature 22±1°. Rates are expressed as in Fig. 8. In the catalytic experiments the conditions were as given in the Experimental section; reaction was begun by the addition of 4·4–6·6 μg. of enzyme. Stopped-flow experiments were performed as described in Fig. 8; final concentration of enzyme in the reaction chamber, 27 μM; ratio of DPNH to enzyme used to preform the FADH' enzyme, 3·0 moles/mole. (B) Results with lipoylglycylglycine (shown on a more convenient scale in Fig. 8) are given for comparison.

mixing with DPNH) had taken place, and so must have been more than 90% complete in less than 3 msec. The minimum turnover number is then greater than 50 000 min.$^{-1}$.

Rates of reoxidation of the red intermediate by oxidized lipoic acids. Previous work by Sanadi, Langley & Searls (1959) and by Massey (1960*a*) had shown that the rates of catalytic oxidation of DPNH by various lipoic acid derivatives varied considerably with the derivative used. In the present work it has been found that there is a close correlation between the rates of the enzyme-catalysed oxidation of DPNH by the lipoyl derivatives used and the rates of reoxidation of the red intermediate (produced by DPNH addition) by the same lipoyl derivatives. This phenomenon is illustrated in Figs. 8 and 9 for DL-lipoylglycylglycine and DL-α-lipoic acid. It may be seen that lipoylglycylglycine reacts about 50 times as rapidly as lipoic acid in the catalytic oxidation of DPNH; similarly, the former compound is about 50 times as fast as the latter compound in reconverting the red intermediate into the oxidized enzyme. In these Figures and following ones, the turnover numbers for the catalytic oxidation of DPNH in the reaction DPNH + H^+ + oxidized lipoic derivative $\rightleftharpoons$ DPN + reduced lipoic derivative are for a two-electron transfer; as will be considered in the Discussion, the numerically similar or identical turnover numbers from the stopped-flow experiments probably represent only a one-electron transfer due to the interconversion of flavinadenine dinucleotide (FAD) and its semiquinone (FADH$^\cdot$).

Kinetics of the reduction of diphosphopyridine nucleotide by dihydrolipoamide. When plots according to the method of Lineweaver & Burk (1934) were made of the effect of DPN concentration on its rate of reduction by dihydrolipoamide, the results shown in Fig. 10 were obtained at different concentrations of dihydrolipoamide. Such kinetics are consistent with the general mechanism given by Alberty (1953) for a two-substrate system in which the enzyme exists in two different states (oxidized and reduced) and where the substrates combine (and react) with particular forms of the enzyme:

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E' + B,$$

$$E' + C \underset{k_6}{\overset{k_5}{\rightleftharpoons}} EC \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E + D,$$

where E represents oxidized enzyme, E$'$ reduced enzyme, A a reducing substrate, B its oxidized form, C an oxidizing substrate, D its reduced form and k_1–k_8 the rate constants of the reactions shown. With this mechanism the limiting rate at infinite concentration of one substrate at any particular concentration of the other may be obtained by Lineweaver & Burk extrapolation; such extrapolated values for infinite concentrations of dihydrolipoamide are shown in Fig. 10 as a function of DPN concentration. In Fig. 11 the extrapolated limiting rates for infinite DPN concentration are plotted as a function of dihydrolipoamide concentration. Under the conditions of salt concentration, pH and temperature given in the legend to Fig. 10, K_m for DL-dihydrolipoamide is 0·3 mM, K_m for DPN is 0·2 mM, and $V_{max.}$ (the limiting rate with infinite concentrations of both substrates) is $3{\cdot}33 \times 10^4$ moles of DPN reduced/min./mole of enzyme.

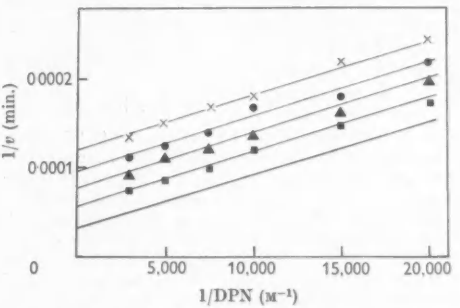

Fig. 10. Effect of DPN and dihydrolipoamide concentrations on the rates of the reaction dihydrolipoamide + DPN → lipoamide + DPNH + H^+. Buffer, 0·05 M-phosphate, pH 7·6; temperature 25°. ×, with 0·1 mM-DL-dihydrolipoamide; ●, with 0·133 mM-DL-dihydrolipoamide; ▲, with 0·2 mM-DL-dihydrolipoamide; ■, with 0·333 mM-DL-dihydrolipoamide. The continuous line without experimental points represents the values obtained by extrapolation to infinite DL-dihydrolipoamide concentration. Reaction was begun by the addition of 0·22 μg. of enzyme.

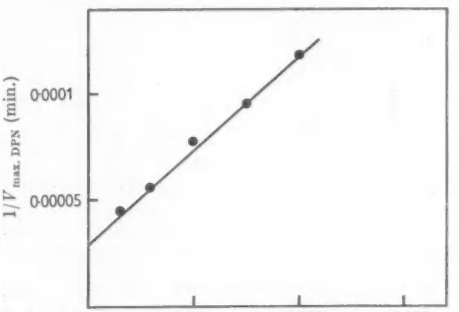

Fig. 11. Effect of DL-dihydrolipoamide concentration on the $V_{max.\ DPN}$ values obtained for the experiment described in Fig. 10.

Rates of red-intermediate formation by reduced lipoic acids. The rates at which the red intermediate is formed by reduced lipoic acids have been determined by the stopped-flow technique under conditions of temperature, pH and salt concentration identical with those of Fig. 10. Results with dihydrolipoamide expressed as turnover numbers are given in Fig. 12, which also shows for comparison the extrapolated turnover numbers (at infinite DPN concentration) obtained from the catalytic experiments described in Figs. 10 and 11. It is evident that the rate of formation of the red intermediate by dihydrolipoamide is the same as that of the overall catalytic reduction of DPN by the compound. A comparison of the time course of the formation of the red colour with a theoretical model is shown in Fig. 14.

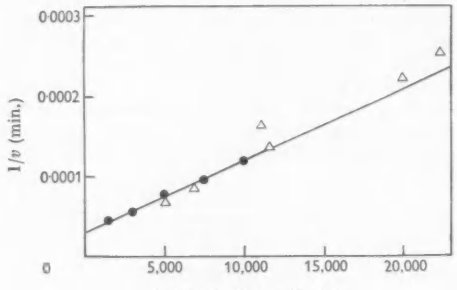

Fig. 12. Comparison of the maximum rates (∞ [DPN]) in the reaction dihydrolipoamide + DPN→lipoamide + DPNH + H^+ (●), and the rates of $FADH^{\cdot}$ formation (△). The latter were measured by the stopped-flow method; the former are the values shown in Fig. 11. In the stopped-flow experiments (△), performed at 25° in 0·05 M-phosphate, pH 7·6, the final concentration of enzyme in the reaction chamber was either 21·5 μM or 12·3 μM; concentrations of DL-dihydrolipoamide were as shown.

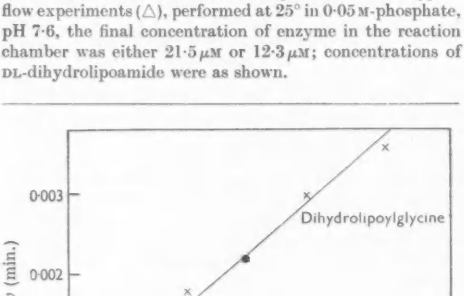

Fig. 13. Effect of DL-dihydrolipoylglycine concentration on the rate of the enzyme-catalysed reaction dihydrolipoylglycine + DPN→ DL-lipoylglycine + DPNH + H^+ (×), and the rate of $FADH^{\cdot}$ formation (●) by dihydrolipoylglycine. Buffer, 0·05 M-phosphate, pH 7·6; temperature 25°. In the catalytic experiments the dihydrolipoylglycine concentration was varied as shown; the DPN concentration used was 0·67 mM. No significant variation in the rates was observed on lowering the DPN concentration to 67 μM. In the stopped-flow experiments the final concentration of enzyme in the reaction chamber was 21·5 μM; concentrations of DL-dihydrolipoylglycine were as shown.

Fig. 13 shows a comparison of the turnover numbers of the catalytic reduction of DPN by another reduced lipoyl derivative, dihydrolipoylglycine, and the turnover numbers obtained by the stopped-flow technique. The results for dihydrolipoamide from Fig. 12 are also included in the figure to illustrate the much greater rates of reaction obtained with the latter substrate. It should be noted that the turnover numbers from the catalytic experiments with dihydrolipoylglycine are actual experimental values, not extrapolated ones as with dihydrolipoamide. When an analysis of the kinetics of the reduction of DPN by dihydrolipoylglycine similar to that for dihydrolipoamide (shown in Figs. 10 and 11) was attempted, the changes in rate on varying the DPN concentration at any fixed concentration of dihydrolipoylglycine were within the limits of experimental error of the measurements.

Rates of reoxidation of the red intermediate by diphosphopyridine nucleotide. When the red intermediate was produced anaerobically by the addition of DL-dihydrolipoamide it was found that the rate of reoxidation by DPN was so fast that it could not be estimated by the stopped-flow technique. Thus in a typical experiment the red intermediate was made anaerobically by the addition of 0·1 mM-dihydrolipoamide to 43 μM-enzyme at pH 7·6, transferred immediately to the stopped-flow apparatus and mixed with 0·4 mM-DPN at 25°. (Note that the concentrations of all reactants are halved on mixing.) The reoxidation under these conditions was complete by the time of first observation (3 msec.), indicating that the disappearance of red colour occurred at a rate giving a turnover number considerably in excess of 5×10^4 min.$^{-1}$.

DISCUSSION

It is known from the work of Kuhn & Wagner-Jauregg (1934), Michaelis *et al.* (1936) and Beinert (1956) that free flavins are reduced by two single-electron reduction steps in which the oxidized flavin is yellow, the half-reduced (or semiquinone or free-radical) form is red and the fully reduced form is nearly colourless. As mentioned in the intro-

duction, several flavoproteins have been reported to form a red colour on the addition of reducing substrate. From the work of Dolin (1956) on DPNH peroxidase it is clear, however, that the mere formation of red colour on the addition of reducing substrate is not necessarily an indication of semiquinone formation. With diaphorase the evidence is nevertheless strongly in favour of the view that the red colour represents a stage in reduction between that of oxidized and reduced forms of flavinadenine dinucleotide. Thus it is produced by the addition of DPNH or reduced lipoic acids and disappears with return of the yellow colour and fluorescence of the oxidized enzyme on the addition of DPN, oxidized lipoic acids, potassium ferricyanide and oxygen. Furthermore, it is produced as an intermediate in the reduction of the enzyme by dithionite, where the final form of the flavin is that of full reduction. Thus by analogy with the previous work on free flavins it should represent the semiquinone or free radical form of the flavinadenine dinucleotide ($FADH^{\cdot}$). Whether a free radical in the conventional sense exists in the protein is still a matter for experiment. The stability of the red colour in the absence of any oxidizing agent is remarkable. The effect of *p*-chloromercuriphenyl sulphonate in discharging the red colour and at the same time causing a general bleaching of the visible absorption (Fig. 7) seems to suggest that the red colour is stabilized by interaction with a protein sulphydryl group. Recent work (Massey & Veeger, 1960) has indicated that in the catalytic action of the enzyme a protein disulphide bridge is converted into a dithiol grouping. As discussed in the last-named paper, a likely sequence of events is that one of the nascent sulphydryl groups of the protein transfers a hydrogen atom and an electron to flavinadenine dinucleotide to convert the latter into $FADH^{\cdot}$; this sulphur atom would thus be left as a free radical. Possibly the stability of the enzyme–$FADH^{\cdot}$ is due to a limited overlap of the electron clouds of the $FADH^{\cdot}$ and the $S^{\cdot}$; this would be disturbed by a mercaptide-forming substance such as *p*-chloromercuriphenyl sulphonate. If this situation existed it is possible that very little free radical might be detected.

A feature of the results is the stoicheiometry of the formation of red colour and the amount of reducing substrate added. The titration data for both DPNH and dihydrolipoamide show that the full change in extinction at 530 mμ is produced by 1 mole of DPNH or 1 mole of the natural isomer of dihydrolipoamide/mole of FAD. If the red colour is due to the conversion of FAD into $FADH^{\cdot}$, as appears likely, it is necessary to account for the fate of the other electron involved in these reactions, and the idea that a protein disulphide–dithiol reaction takes part in the electron exchange provides a reasonable explanation for the phenomenon.

A comparison of the rates of catalytic action under a variety of conditions and the rates of formation and reoxidation of the red intermediate (Figs. 8–14) shows that the red form is a true intermediate in the enzyme action. In these figures the rate constants for the overall catalysis (a two-electron change) are the same as those for the formation or oxidation of the red intermediate. If the intermediate is a semiquinone, then this one-electron change occurs at the same rate as the two-electron-change enzyme catalysis. Thus the rate determinations are consistent with the titration data of Figs. 3 and 4, indicating that the second electron is donated or accepted by another group in the protein. Further, the rate of conversion of FAD into $FADH^{\cdot}$ appears to be the rate-determining step in the catalytic reduction of DPN by dihydrolipoic acids, and the reoxidation of $FADH^{\cdot}$ by oxidized lipoic acids the rate-determining step in the catalytic oxidation of DPNH. This conclusion is supported by the extremely high rates of $FADH^{\cdot}$ formation by DPNH and the reoxidation of $FADH^{\cdot}$ by DPN which could be observed, but which were too fast to measure by the stopped-flow method. This conclusion is also supported strongly by a consideration of the kinetics of the enzyme-mediated reaction between DPN and dihydrolipoamide. The results presented in Figs. 10 and 11 may be treated in terms of the scheme given by Alberty (1953), which is reproduced in the Results section. In this particular case dihydrolipoamide is A in the scheme and DPN is C. Then the concentration of dihydrolipoamide required to allow the

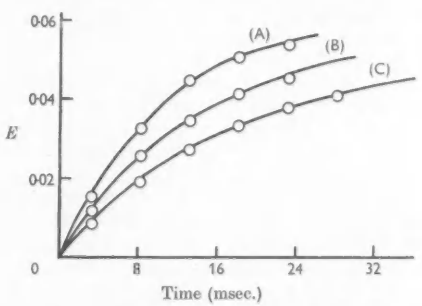

Fig. 14. Comparison of the observed changes in extinction at 530 mμ, on mixing diaphorase and DL-dihydrolipoylglycine, with the calculated values. Concentration of diaphorase 12·3 μM after mixing; concentrations of DL-dihydrolipoylglycine: (A) 75 μM, (B) 50 μM, (C) 35 μM; 0·05 M-phosphate buffer, pH 7·6, at 25°; 2 cm. light path. Lines were calculated as described in the text.

reaction to proceed at half-maximum speed (with infinite DPN concentration) may be written

$$K_{\text{dihydrolipoamide}} = [k_7(k_2+k_3)]/[k_1(k_3+k_7)],$$

and the corresponding quantity for DPN is

$$K_{\text{DPN}} = [k_3(k_6+k_7)]/[k_5(k_3+k_7)].$$

The maximum rate, with infinite concentrations of both substrates, is $V_{\text{max.}} = k_3k_7/(k_3+k_7)$. Now, the value of $V_{\text{max.}}$ in the experiments of Fig. 10 with catalytic quantities of enzyme is

$$3{\cdot}33 \times 10^4 \text{ min.}^{-1};$$

this result agrees within experimental error with the rate obtained in stopped-flow experiments on the formation of $FADH^{\cdot}$ from FAD by dihydrolipoamide, when extrapolated to infinite substrate concentration (Fig. 12). This maximum rate may, since no DPN was present in the stopped-flow experiments and k_7 does not come into consideration, be equated with k_3. Then k_3 (stopped flow) $= k_3k_7/(k_3+k_7)$ (catalytic), requiring that $k_7 \gg k_3$, in agreement with the finding that the rate of reoxidation of $FADH^{\cdot}$ by DPN is too rapid to measure by the stopped-flow method. Further, when no DPN is present, $K_{\text{dihydrolipoamide}}$ becomes $(k_2+k_3)/k_1$, whereas in the presence of DPN this expression must be multiplied by $k_7/(k_3+k_7)$ to give the formula already quoted. It follows that the agreement between the catalytic and stopped-flow measurements of $K_{\text{dihydrolipoamide}}$ shown in Fig. 12 is again consistent with a value of $k_7 \gg k_3$.

As a further test of the validity of the above analysis of the kinetics, the theoretical time course of $FADH^{\cdot}$ formation was calculated from the kinetic constants obtained from Figs. 10 and 11 and the extinction coefficient $E_{530\ \text{m}\mu}$ for $FADH^{\cdot}$ formation given by the titration experiment with DL-dihydrolipoamide shown in Fig. 4. In Fig. 14 the theoretical time course of extinction changes at 530 mμ for three different concentrations of dihydrolipoamide is compared with the values actually observed in stopped-flow experiments. This figure provides a striking demonstration of the role of the red intermediate in the overall catalytic reaction, since the curves were calculated without disposable constants.

The conversion of the oxidized enzyme into the semiquinone in the stopped-flow experiments may be written:

$$dy/dt = k_2x - k_1y(a+y),$$
$$dx/dt = k_1y(a+y) - (k_2+k_3)\,x,$$

where y is the concentration of oxidized enzyme, a is the difference between the concentration of substrate and of enzyme, x is the concentration of enzyme–substrate complex and k_1, k_2 and k_3 have the same meanings as in the analysis of the preceding paragraphs. To obtain numerical values, k_3 is taken to be equal to $V_{\text{max.}}$, and a minimum value for k_2 of 1300 sec.$^{-1}$ is derived from catalytic studies of the reaction

lipoamide + DPNH + H^+ → DPN + dihydrolipoamide.

With this value of k_2 and the value of $K_{\text{dihydrolipoamide}}$ from Fig. 12, $k_1 = 1{\cdot}24 \times 10^7$ M^{-1} sec.$^{-1}$. Numerical calculation with these values yields the excellent agreement of Fig. 14 for the time course of the appearance of the semiquinone, thus supporting further the arguments based on the initial rates of colour formation at 530 mμ. Although the value assigned to k_2 is a lower limit rather than an estimate of its actual value, this in no way affects the meaning of the agreement of Fig. 14, because increasing the value of k_2 [with corresponding adjustment of k_1 to maintain $(k_2+k_3)/k_1$ equal to $K_{\text{dihydrolipoamide}}$ influences the course of colour formation significantly only in the first 2–3 msec., i.e. before observation by the stopped-flow method can begin.

Beinert (1957) has presented evidence that changes involving coloured intermediates of flavoprotein enzymes can take place fast enough to admit of their participation in the enzyme catalytic mechanisms. We believe that the experiments reported here constitute a convincing demonstration that these coloured compounds not only can but do function as intermediates in catalysis.

SUMMARY

1. The anaerobic addition of either reduced diphosphopyridine nucleotide or dihydrolipoic acid derivatives to diaphorase results in the production of a red colour, characterized by an increase in extinction in the 500–650 mμ region of the spectrum, and changes in other regions of the visible spectrum.

2. The effect of diphosphopyridine nucleotide, oxidized lipoic acids, potassium ferricyanide, oxygen and dithionite on the red form of the enzyme indicate that it represents an intermediate oxidation level of the enzyme-bound flavinadenine dinucleotide between the fully oxidized and fully reduced forms. By analogy with previous work, this form has been ascribed to the semiquinone or free radical state of the flavin ($FADH^{\cdot}$).

3. The effect of *p*-chloromercuriphenyl sulphonate on the red form is described; the results indicate that the semiquinone form is stabilized by interaction with a protein sulphydryl group, possibly a sulphur radical.

4. The rates of formation of the red intermediate and of its reoxidation under a variety of conditions have been studied by the stopped-flow method. Comparison of the rates obtained with catalytic rates estimated under the same conditions shows

that the rate of formation of FADH$_2$ by dihydrolipoic acids is the rate-determining step in the catalytic reduction of diphosphopyridine nucleotide by dihydrolipoic acids. Similarly, the rate of reoxidation of FADH$_2$ by oxidized lipoic acids is the rate-determining step in the catalytic oxidation of reduced diphosphopyridine nucleotide by oxidized lipoic acids. The results of the rate experiments and titration experiments show that in the overall two-electron oxidation–reduction reaction catalysed by the enzyme the flavinadenine dinucleotide is reduced to FADH$_2$ and reoxidized only once per catalytic cycle. The relation of this finding to the reaction mechanism of the enzyme is discussed.

We are indebted to Dr I. C. Gunsalus and Dr L. J. Reed for generous gifts of DL-lipoic acid and lipoyl derivatives and to Miss M. Hamshaw for the preparation of large amounts of enzyme. One of us (C.V.) is an O.E.E.S. Senior Visiting Fellow, working under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.), with financial aid from the Netherlands Organization for the Advancement of Pure Research. The equipment used was largely provided by the Medical Research Council and the Rockefeller Foundation.

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The Hydrolysis of Dipeptides by Different Regions of Rat Small Intestine

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Recent studies of the small intestine have shown that its absorptive capacity for different nutrients varies according to the region. In the rat, Fisher & Parsons (1950*b*), showed that glucose is most rapidly absorbed in the duodenum and this is also reported to be the most active area for the absorption of iron (Brown & Justus, 1958). On the other hand, the distal half of the small intestine in the rat seems to be the site of maximal absorption of vitamin B_{12} (Coates & Holdsworth, 1958), of ^{131}I-labelled fat (Benson, Chandler, Vansteehuyse & Gagnon, 1956) and of water (Barry, Matthews & Smyth, 1959). Kremens, Linner & Nelson (1954) have shown, using dogs, that the distal half of the small intestine is more important than the proximal half in maintaining fat balance.

Information on the site of absorption of protein breakdown products, i.e. peptides and amino acids, is scanty. Neil (1959) has reported that, in the rat, the rates of absorption of L-cystine and L-cysteine are highest in the duodenum, decrease in the jejunum and are lowest in the ileum; Korelitz & Frank (1959) have shown that translocation of

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alanine in guinea pig occurs more swiftly in the jejunum than in the ileum. Experiments *in vitro* on the absorption of peptides have shown that small quantities of glycylglycine can penetrate through the intestine (Agar, Hird & Sidhu, 1953; Newey & Smyth, 1959*a*; Wiggans & Johnston, 1959), as can traces of L-leucylglycine (Agar, Hird & Sidhu, 1953). Further, the evidence of Florey, Wright & Jennings (1941) indicates that the peptidases of the succus entericus are confined to cells desquamated from the mucosa. These findings suggest that peptide hydrolysis may occur primarily inside the mucosal cell rather than in the intestinal lumen, a possibility stated by Newey & Smyth (1959*b*).

It was of interest, therefore, to investigate the distribution of peptidase activity in small intestine, to further the understanding of the processes involved in protein digestion and absorption.

A preliminary report of the present work has appeared elsewhere (Shaw & Robinson, 1958).

MATERIALS AND METHODS

Dipeptides. These were obtained commercially and were checked for purity by paper chromatography before and after acid hydrolysis. Where necessary these were purified by recrystallization.

Animals. Adult, male, albino rats of the Glaxo strain were used; the average weight was 250 g. They were allowed only water for 16 hr. before use, to ensure an empty intestine.

Enzyme extracts. All operations were carried out at 4°. The animal was killed by a blow on the head, the small intestine rapidly dissected out and divided into duodenum, jejunum and ileum. For the purposes of characterization, the duodenum was taken as the section of intestine from the pylorus to the ligament of Treitz, the jejunum as the proximal third of the remaining small intestine and the ileum as the distal two-thirds.

The central inch of each of these sections was cut out and slit longitudinally to expose the mucosal surface. This surface was then washed quickly with a fine jet of distilled water to remove adhering mucus, and the mucosa was scraped away with a scalpel. Histological examination showed that all the mucosa was removed by this procedure leaving only a thin layer of submucosa attached to the musculature. The mucosal scrapings from each section were then separately homogenized with a known volume of water in a Potter–Elvehjem-type homogenizer. The volume of distilled water used, 5–10 ml., was determined by preliminary experiments, to give a convenient enzyme concentration for assay. The homogenates were left for 2 hr. to allow complete extraction of the enzymes from the cell debris. They were then centrifuged for 20 min. at 26 000 *g* in a refrigerated centrifuge, and the supernatant was used as the enzyme extract. The pH throughout this process was approximately 6·5.

This method of extraction was compared with extractions with 10% glycerol or dilute buffers at different pH values and was as effective as, but no better than, these. The residue showed no activity with dipeptide substrates, nor was any obtained by further extraction with water.

Hydrolysis of dipeptide. The ninhydrin method of Moore & Stein (1948) was used and the increase in final colour was the basis for estimating the degree of hydrolysis of the dipeptides. However, the colour yield is not linearly related to the α-amino nitrogen when the extinction exceeds 0·5 (Moore & Stein, 1948), and, in addition, the colour yields of some peptides do not correspond with the theoretical ones (Yanari, 1956). Accordingly, for each dipeptide used, a standard curve was constructed by using mixtures of the dipeptide and its constituent amino acids; this related the colour yield (measured as extinction), to the degree of hydrolysis of the peptide.

In samples of the reaction mixture used in each assay, small amounts of protein were present from the enzyme extract. Preliminary experiments showed that the colour given by this protein with the ninhydrin reagent was negligible and did not interfere with the assay.

Determination of enzyme activity. As the intestinal mucosa contains many enzymes which hydrolyse peptides, preliminary experiments were performed to ascertain the optimum conditions for measurement of enzyme activity. Although the term 'peptide-splitting activity' has been used, it is possible that, under the conditions of assay, more than one enzyme is involved.

Measurements of peptidase activity were made as follows: equal volumes (0·08 ml.) of 0·2 M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, at the optimum pH of the activity being measured, and 0·0625 M-dipeptide solution were incubated together at 37° in a small tube sealed with a rubber stopper. After 5 min., 0·04 ml. of enzyme extract was added, the tube was shaken quickly and returned to the water bath. Two samples of 0·01 ml. were withdrawn immediately and pipetted into test tubes containing 1 ml. of ninhydrin reagent which stopped the reaction. Similar samples were withdrawn at known time intervals in a 1 hr. period and the degree of hydrolysis was measured. All volumes were measured with an Agla micro-syringe (Burroughs Wellcome and Co. Ltd.).

Blank determinations carried out with boiled enzyme alone, dipeptide alone, and dipeptide together with boiled enzyme, showed no increase in ninhydrin colour.

When metal ions were used in an attempt to activate the enzyme extract, the metal ion was added to the reaction mixture in the buffer; molarities stated refer to the final molarity in the reaction mixture.

The final molarity of the substrate in the reaction mixture was, in all cases, 0·025 M.

The extracts of the three sections from one animal were assayed concurrently.

Units of peptide-splitting activity. One unit represents the hydrolysis of 1 mole of substrate in 10 min. Results are expressed as units of activity/mg. of protein.

Determination of protein. Protein nitrogen was determined by the micro-Kjeldahl method; protein was calculated as mg. of $N \times 6{\cdot}25$.

RESULTS

Characterization of peptide-splitting activities

L-*Leucylglycine.* Enzymes hydrolysing this substrate are widely distributed and have been demonstrated in rat intestinal mucosa by Berger & Johnson (1940). These authors showed that maxi-

mum activity occurred at pH 8·6–8·8 and that, after dialysis, activation by Mg^{2+} and Mn^{2+} ions could be obtained. The crude preparation studied in our experiments had a pH optimum at pH 8·5–8·6 (Fig. 1) and activity was decreased by 20% when 0·01 M-Mg^{2+} was added to the reaction mixture. Therefore assays were carried out at pH 8·6 without added metals. Activity was quite stable, only decreasing by 40% after three days at 0°; this was in contrast to the instability of the other enzymes studied.

The reaction followed zero-order kinetics and the rate of reaction was proportional to enzyme concentration (Fig. 2).

Glycylglycine. An enzyme, hydrolysing glycylglycine, was demonstrated in rat intestinal mucosa by Berger & Johnson (1940). A similar enzyme was found in rat muscle by Smith (1948*a*); this enzyme was activated by Co^{2+} ions, and to a lesser extent by Mn^{2+} ions at pH 8·0.

The extracts studied by us had quite low activity when no metals were added, but activity was increased about threefold by mM-Co^{2+}. The activated preparation had two pH optima, at 7·4 and at 8·2 (see Fig. 1). Activity at pH 7·4 was decreased by 10% with mM-Mn^{2+}, but enhanced about threefold by mM-Co^{2+}. At pH 8·2 activity was similarly increased by mM-Co^{2+} but was also

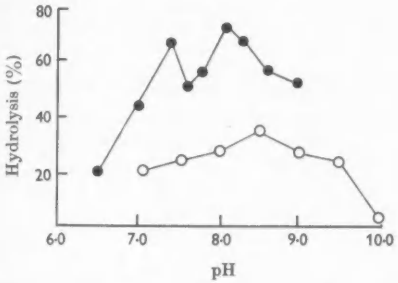

Fig. 1. pH optima for the hydrolysis of dipeptides by mucosal extracts. ●, Glycylglycine; ○, L-leucylglycine. Reaction mixtures contained 0·08 ml. of 0·2 M-tris buffer, 0·08 ml. of 0·0625 M-dipeptide, 0·04 ml. of mucosal extract; incubated for 30 min. at 37°. For glycylglycine the reaction mixture contained 0·2 μM-Co^{2+}.

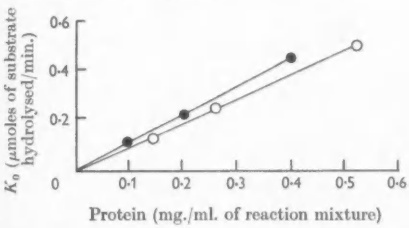

Fig. 2. Plot of zero-order rate constants (K_0) against protein content of the reaction mixture for the hydrolysis of dipeptides by mucosal extracts. ●, Glycylglycine (measured at pH 8·2); ○, L-leucylglycine (measured at pH 8·6) K_0 = μmoles of substrate hydrolysed/min. Reaction mixture contained 0·08 ml. of 0·2 M-tris buffer, 0·08 ml. of 0·0625 M-dipeptide and 0·04 ml. of mucosal extract. For glycylglycine the reaction mixture contained 0·2 μM-Co^{2+}. Temp. 37°.

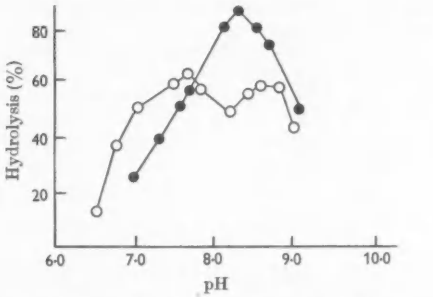

Fig. 3. pH optima for the hydrolysis of dipeptides by mucosal extracts. ●, Glycyl-L-alanine; ○, glycyl-L-leucine. Conditions as for Fig. 1.

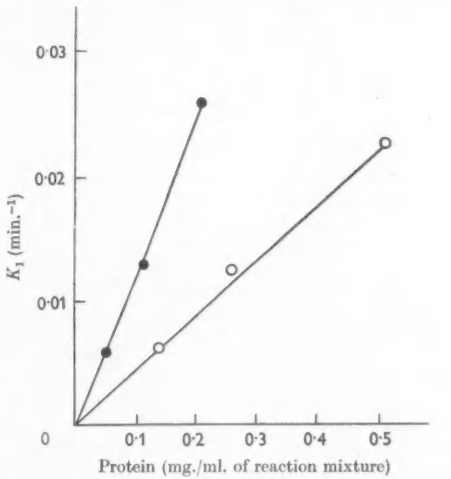

Fig. 4. Plot of first-order rate constant (K_1) against protein content of the reaction mixture for the hydrolysis of dipeptides by mucosal extracts. ●, Glycyl-L-leucine (measured at pH 8·6); ○, glycyl-L-alanine (measured at pH 8·3). Values for K_1 are derived from a graph of log $S/(S-x)$ against time where S is the initial substrate concn. and x is the concn. after a known time for each reaction. Reaction conditions as for Fig. 2.

increased two and a half times by mM-Mn^{2+}, indicating that two enzymes were present.

Measurement of the distribution along the intestine was carried out at pH 8·2 in the presence of mM-Co^{2+}.

The reaction was zero order, and the rate of peptide splitting was proportional to enzyme concentration (see Fig. 2).

Glycyl-L-leucine. This was shown to be hydrolysed by an enzyme from rat muscle by Smith (1948*b*). The enzyme retained its activity well at 0°, and was activated by Zn^{2+} in the presence of phosphate ions.

Activity in intestinal mucosa was maximal at two pH optima, 7·6 and 8·6 (see Fig. 3). At pH 7·6, the crude extract was inhibited by mM-Co^{2+}, -Mg^{2+}, -Mn^{2+} and -Zn^{2+}, to the extent of 70, 20, 50 and 90% respectively. At pH 8·6, the corresponding inhibitions were 25, 35, 25 and 30%. Contrary to the findings of Smith, inhibition with Zn^{2+} occurred, even when 0·2 M-phosphate buffer was used. Activity, at both pH optima, was very labile, 50–60% being lost after 16 hr. at 0°; some preparations lost all their activity after this treatment.

Assays on distribution were performed at pH 8·6 without added metals.

Hydrolysis followed first-order kinetics, and the rate was proportional to enzyme concentration (Fig. 4).

Glycyl-L-alanine. Price, Meister & Greenstein (1949) showed that this peptide was hydrolysed by rat kidney.

Activity of the mucosal extract had one pH optimum at 8·3 (Fig. 3) and was inhibited by 0·001 M-Co^{2+}, -Mg^{2+}, -Mn^{2+}, and -Zn^{2+}. It was extremely labile, all activity being lost when the extract was kept at 0° overnight. The lability

Table 1. *Distribution of peptide-hydrolysing activity in rat small intestine*

1 unit is equivalent to the hydrolysis of 1 μmole of substrate in 10 min. Levels of activity in: D, duodenum; J, jejunum; I, ileum.

Substrate	Expt. no.	Activity (units/mg. of protein) Duodenum	Jejunum	Ileum
L-Leucylglycine	1	13·2	12·7	19·7
	2	9·8	15·0	13·7
	3	8·4	10·9	12·6
	4	5·6	9·2	15·8
	5	1·8	3·14	14·0
	6	5·8	6·93	9·2
	Mean	7·4	9·6	14·1
P (D – J) > 0·1		P (J – I) < 0·1	P (D – I) = 0·01	
Glycylglycine	1	3·0	8·6	9·5
	2	0·9	1·33	7·7
	3	7·5	7·60	9·1
	4	6·1	6·78	12·5
	5	4·9	6·8	11·9
	6	5·9	11·1	11·4
	Mean	4·7	7·0	10·31
P (D – J) > 0·1		P (J – I) < 0·05	P (D – I) = 0·001	
Glycyl-L-leucine	1	18·2	21·8	30·7
	2	13·8	38·0	36·0
	3	21·4	27·9	42·8
	4	16·2	28·8	26·6
	5	14·4	24·8	39·9
	6	14·1	20·0	23·2
	7	28·8	52·5	68·2
	Mean	18·1	30·5	38·2
P (D – J) < 0·05		P (J – I) > 0·1	P (D – I) < 0·01	
Glycyl-L-alanine	1	12·05	19·6	23·5
	2	18·9	18·4	27·0
	3	14·1	26·0	38·9
	4	11·9	18·5	29·6
	5	20·0	20·0	20·8
	6	18·0	25·2	25·3
	Mean	15·8	21·3	27·5
P (D – J) > 0·1		P (J – I) = 0·05	P (D – I) < 0·01	

suggested that the enzyme responsible for the hydrolysis of glycyl-L-alanine was identical with that for glycyl-L-leucine, but after 14 hr. at 0°, activity against glycyl-L-leucine decreased by 50 % whereas that against glycyl-L-alanine decreased by 80 %, indicating different enzymes.

The activity was sufficiently labile at 37° to decrease during the reaction period. This was reflected in the plot of log $[S/(S-x)]$ against time (where S is the initial concentration and x is the concentration after a known time), giving a curve instead of the expected straight line. Rate measurements were taken from the tangent to the curve at the origin, and were proportional to enzyme concentration (see Fig. 4). If high concentrations of extract were used, a straight line could be obtained but a high concentration could not always be obtained in distribution experiments because of variations in the mucosa; the tangent method was therefore used.

Assays were carried out at pH 8·3 without any activation.

Distribution of peptide-splitting activity

There was an increase in peptide-splitting activity along the intestine; it was lowest in the duodenum and became progressively higher toward the ileum (Table 1). The difference between the levels of activity in duodenum and ileum is significant, $P \leqslant 0{\cdot}01$. With glycyl-L-alanine and glycylglycine, the differences between ileum and jejunum are significant, $P = 0{\cdot}05$ and $P < 0{\cdot}05$ respectively, but the differences are not as clear as for the other two peptides.

The differences between duodenum and jejunum are only significant in the case of glycyl-leucine.

Experiments performed on consecutive sections of ileum showed that activity in any one region was not abruptly different from that of the neighbouring regions; extracts of four consecutive inch samples showed activities of 13·0, 14·0, 14·5, 13·0 units/mg. of protein. This indicates that the change in peptidase activity is a gradual one and does not vary at random.

Table 2. *Protein content of different regions of the intestine*

Total protein was extracted from 1-in. segments of rat small intestine.

Expt. no.	Protein (mg.) Duodenum	Jejunum	Ileum
1	8·2	8·7	5·7
2	9·5	10·0	7·9
3	7·9	9·0	8·1
4	7·5	7·2	8·2
5	7·5	8·9	8·8
6	7·1	7·7	6·0
7	7·1	6·5	6·5
8	9·0	9·1	9·1
Mean	7·97	8·3	7·5

$P > 0{\cdot}1$ for all cases.

Peptidase activities were related to protein content and not to serosal length as the latter is difficult to measure accurately. There remained the possibility that there is a variation in protein content along the intestine, which would cause ambiguous results. It has been demonstrated by Wood (1944) and Fisher & Parsons (1950*a*) that there is a greater mucosal area per unit of intestinal length in the jejunum of rat than in the ileum. Accordingly we estimated the amount of protein obtained from samples of intestinal tissue used for preparation of enzyme extracts and compared them (Table 2). These results showed no significant difference of protein content per unit of serosal length in the different regions of the intestine. There is a high variance due to individuality and difficulty in measuring the length of the section.

DISCUSSION

Although optimum conditions for each peptide were used, this does not ensure that only one enzyme is being measured in the crude mucosal extract and indeed it is likely that activity is a result of the simultaneous action of a number of enzymes. The results obtained here are, therefore, a measure of the distribution of the ability of the intestine to hydrolyse peptides presented to it, rather than the distribution of particular enzymes. The results obtained support other experimental data suggesting that the intestine does not have a constant function along its length, but that there are variations of function according to region.

Fisher (1954) has suggested that protein may be partly absorbed as peptides. Recent investigations have shown that when the mucosa, *in vitro*, is presented with peptides, the constituent amino acids appear on the serosal side of the intestine, although small amounts of glycylglycine (Agar, Hird & Sidhu, 1953; Newey & Smyth, 1959*a*; Wiggans & Johnston, 1959), and L-leucylglycine (Agar, Hird & Sidhu, 1953) are translocated. Newey & Smyth (1959*b*) have further demonstrated that peptides, as such, enter the mucosa. It seems likely that the peptidases of the intestine are associated with this process. The high level of activity in the ileum may indicate a site of maximal absorption.

The amino acids, alanine, cystine and cysteine, are most rapidly absorbed in the upper intestine where peptidase activity is low. Peptidases may be involved in this but may not limit absorption. Absorption of amino acids is dependent upon the respiratory function (Gibson & Wiseman, 1951; Wiseman, 1953; Friedhandler & Quastel, 1955),

and this may be the limiting factor. It is known that respiratory function of the small intestine varies along the intestine, the upper intestine having highest activity (Dickens & Weil-Malherbe, 1941; Fisher & Parsons, 1950*b*; Wilson & Wiseman, 1954).

Peptidases may be concerned with protein synthesis in the intestine as in the root tip of barley (Linderström-Lang, 1952). However, the incorporation of ^{35}S-labelled methionine into intestinal mucosa occurs mainly in the duodenum and the upper ileum (Friedburg, Tarver & Greenberg, 1948). These sites are not coincident with the general region of maximal peptidase activity, which suggests that, in the intestine, the function of peptidases is concerned with digestion and absorption rather than with protein synthesis.

SUMMARY

1. Optimum conditions for the hydrolysis of L-leucylglycine, glycylglycine, glycyl-L-leucine and glycyl-L-alanine, by rat mucosal extracts, have been studied.

2. For all these dipeptides, the highest hydrolytic activity was found to occur in the ileum.

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Biochem. J. (1960) **77**, 356

Metabolism of D- and L-[3-^{14}C]Glycerate by Liver Tissue of the Rat

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It was recently reported (Dickens & Williamson, 1959) that isotopically labelled hydroxypyruvate is well incorporated into liver glycogen by the intact rat and into glucose by rat-liver slices *in vitro*. Possible enzymic routes for this incorporation were suggested, including the possibility that the hydroxypyruvate might first be reduced to glyceric acid. This could either be L-glycerate, known to be produced from hydroxypyruvate by reduced diphosphopyridine nucleotide acting together with the L-lactic dehydrogenase of animal tissues, or D-glycerate produced by an animal enzyme similar to the D-glycerate dehydrogenase which Stafford, Magaldi & Vennesland (1954) discovered in plant

tissues. These possibilities and others are further considered in the Discussion to the present paper.

The most direct approach to this problem was to study the metabolic fate of labelled D- and L-glycerate in liver tissue. This work is reported below.

EXPERIMENTAL

Materials

Radioactive substances. These were obtained from The Radiochemical Centre, Amersham, Bucks, with the following approximate specific activities. Generally labelled [^{14}C]glucose, 10 mc/m-mole; [6-^{14}C]glucose, 2 mc/m-mole; L-[3-^{14}C]serine, 3 mc/m-mole; DL-[3-^{14}C]serine, 1 mc/m-mole. They were diluted as required with non-isotopic material.

Enzymes. D-Glycerate dehydrogenase was prepared from parsley leaves (Stafford *et al.* 1954; cf. Dickens & Williamson, 1958*b*). D-Amino acid oxidase was prepared from pig kidney [Negelein & Brömel (1939) purified to completion of stage 2]. D-Glycerate kinase was prepared from rat liver (Ichihara & Greenberg, 1957). Liver alcohol dehydrogenase was purchased from Boehringer und Söhne, Mannheim.

Methods

These were as described by Batt, Dickens & Williamson (1960*a*) with the following additions.

Glyceric acid was determined either by colorimetric estimation of formaldehyde liberation from C-3 by periodate or by the colour reaction with naphtharesorcinol in nearly conc. H_2SO_4 (Rapoport, 1936; see Dickens & Williamson, 1958*b*). Later the modification of Bartlett (1959) was also used for this purpose. Recrystallized barium DL-glycerate was used as a standard after quantitative conversion into the sodium salt by Na_2SO_4.

Degradation of radioactive substances. The general methods described by Batt, Dickens & Williamson (1960*a*) were followed. Glucose was precipitated from the completely deionized solutions as glucosazone, which was counted for ^{14}C content, recrystallized, re-counted and degraded by the periodate oxidation method. Bacterial degradation of the glucose molecule was also carried out, by fermentation to two molecules of lactate by means of *Lactobacillus casei* (for a full description see Bernstein & Wood, 1957). For this purpose, the deionized medium was concentrated *in vacuo* and the radioactive glucose purified by paper chromatography in ethyl acetate–pyridine–water before fermentation (cf. Batt *et al.* 1960*a*). This method gives the total radioactivity in each of the following pairs of glucose carbon atoms: C-1 + C-6, C-2 + C-5 and C-3 + C-4. On the other hand, periodate degradation of the glucosazone gives the labelling in C-1 + C-2 + C-3; C-4 + C-5; and C-6. Therefore by considering together the degradation by these two methods a more complete picture of the distribution of radioactivity in the glucose molecule is obtainable (cf. Batt, Dickens & Williamson, 1960*b*).

[3-^{14}C]Glyceric acid degradation was by means of periodate oxidation, distillation of the [^{14}C]formaldehyde liberated from C-3 of the acid into dimedone, and measurement of radioactivity of the crystalline formaldehyde–dimedone (cf. Batt *et al.* 1960*a*). Unlabelled carrier DL-glycerate was added where necessary.

The methods of measuring the radioactivity of glucosazone, of $^{14}CO_2$ as $Ba^{14}CO_3$, and of [^{14}C]formic acid as $Ba^{14}CO_3$, were also as described by Batt *et al.* (1960*a*).

Conditions of animal experiments. These were similar to those of Dickens & Williamson (1959) and Batt *et al.* (1960*b*). Batches (1 g.) of slices from the livers of fed rats were incubated aerobically in bicarbonate–saline for 90 min. as these authors described, but the substrate consisted of D- or L-[3-^{14}C]glycerate, usually about 5–10 μmoles containing 0·5–1·0 μc, prepared as described below. After the incubation the total CO_2 was liberated by excess (2 ml. 20%, w/v) of trichloroacetic acid and collected and weighed as $BaCO_3$, of which the radioactivity was determined as usual. The deproteinized extract and washings from the slices and incubation medium were made up to 20 ml. (extract *A*). To 2 ml. of this extract, 100 μmoles of DL-glycerate were added for periodate oxidation, with collection and counting of the liberated formaldehyde as the dimedone derivative. The original solution was similarly assayed and the percentage recovery of the [3-^{14}C] of the added glyceric acid was calculated on this basis.

In order to cover the possible conversion of part of the glyceric acid into [3-^{14}C]serine, which also liberates the terminal carbon atom as formaldehyde on treatment with periodate, the remainder (18 ml.) of the deproteinized extract was passed through a column of Amberlite IR-120 (H^+ form) and washed through with water (total eluate *B*, 25 ml.). Aq. 2N-NH_3 soln. (25 ml.) was then run through and the combined eluate and water washing (*C*) concentrated *in vacuo* to 2·0 ml. To measured samples of extracts *A*, *B* and *C*, 100 μmoles of unlabelled carrier DL-serine was added before periodate oxidation. In some experiments, 50 mg. of DL-serine was first added to *A* and the recovered serine fraction (*C* above) was then recrystallized from 60% ethanol–water before periodate degradation. With this procedure recoveries of radioactivity, from standard solutions of glyceric acid and serine in fractions *B* and *C* respectively, were satisfactory.

Eluate *B* also contains radioactive glucose, in experiments with liver slices. Since periodate would also liberate formaldehyde from this glucose, the sugar was removed, before the periodate analysis for the glyceric acid recovery, by the following method. Eluate *B* was shaken for 2 hr. with Bio-Deminrolit G (see Batt *et al.* 1960*a*) when the originally strongly acidic pH had become 5. The volume of filtrate and washings was noted, and 1·0 ml. was removed for estimation of glucose by the Nelson (1944) method; to the remainder a measured amount of glucose was added, and the solution was concentrated *in vacuo* to small volume. This was either run on paper for purification of glucose for fermentation with *L. casei* (see above) or, if the osazone was to be prepared, it was made up to 10 ml. with water and heated with phenylhydrazine etc. as described by Batt *et al.* (1960*a*). The resulting glucosazone was counted at infinite thickness on a planchet, recrystallized and recounted, and finally degraded by periodate oxidation. Recoveries of radioactivity in the glucose fraction were based on these counts and also on persulphate (total) oxidation of a measured portion of eluate, with subsequent counting as $BaCO_3$. The general precautions detailed by Topper & Hastings (1949), including correction for back-scatter by $BaCO_3$, were followed in all experiments.

Preparation of barium D-*glycerate*

D-Glyceraldehyde, $[\alpha]_D + 16°$, was purchased from Fluka A.G., Buchs, Switzerland, and was oxidized by Br_2 in aqueous solution to D-glyceric acid (Baer, Grosheintz & Fischer, 1939). From the resulting solution the barium salt was prepared, recrystallized three times from aqueous ethanol, and dried over P_2O_5 in a vacuum desiccator until anhydrous. The optical rotation of glyceric acid is unreliable as an indication of optical purity (cf. Rodd, 1952), but $[\alpha]_D$ for the above barium glycerate was $+9·9°$ [*c*, 5·78%, 2 dm. in water; Meyerhof & Schulz (1938) give $+11·5°$]. The addition of ammonium molybdate (final concn. 8·3%) to the neutral sodium salt, prepared quantitatively from the above barium salt by Na_2SO_4 addition, gave $[\alpha]_D + 120°$ calc. for sodium glycerate ($+88·5°$ calc. as barium glycerate). Meyerhof & Schulz (1938) give $[\alpha]_D + 104°$ (basis of calculation not stated); Fager & Rosenberg (1950) found for the sodium salt, molybdate-enhanced, $[\alpha]_D + 83°$, and Stafford *et al.* (1954) give $+75°$ and $+82°$.

Preparation of L-[3-^{14}C]*glyceric acid*

L-[3-^{14}C]Serine (10 mg. containing 20 μc, diluted with 40 mg. of unlabelled L-serine; total 475 μmoles) was deaminated by nitrous acid (Fischer & Jacobs, 1907) to give the corresponding glyceric acid. A parallel experiment was performed with unlabelled L-serine (50 mg.). Both products were passed through Amberlite IR-120 (H^+) columns and washed through with water to 20 ml., concentrated *in vacuo* to syrups, redissolved in 10 ml. of water and reconcentrated to syrups.

The resulting material was passed through Solka Floc cellulose columns, 1 cm. × 15 cm., which had previously been equilibrated with the solvent used [ethanol–aq. NH_3 soln. (sp.gr. 0·88)–water (8:1:1, by vol.)]. Fractions (32) of 3 ml. each were collected, most of the glyceric acid (naphtharesorcinol test) being located in fractions 4–8. These fractions were combined, treated with a little activated charcoal and concentrated to dryness *in vacuo*; the residue (188 μmoles of glyceric acid) was made up to 1·00 ml. for polarimetry in the case of the unlabelled material. Found $[\alpha]_D - 116°$, calc. as the sodium salt, molybdate-enhanced rotation.

The labelled sample, treated exactly similarly, yielded 170 μmoles of L-glyceric acid, but with this material ascending chromatography on paper in the above solvent mixture showed the presence of a trace of a minor radioactive component which moved slightly ahead of the glycerate. No attempt was made to remove this in view of its small contribution to the total radioactivity. Determination of ^{14}C in the formaldehyde liberated from C-3 of the glyceric acid by periodate gave a total recovery of 5·6 μc/170 μmoles of L-glyceric acid.

Preparation of D-[3-^{14}C]*glyceric acid*

DL-[3-^{14}C]Serine was treated with D-amino acid oxidase, the resulting L-[3-^{14}C]serine and hydroxy[3-^{14}C]pyruvate were purified as described in detail by Batt *et al.* (1960*b*). The labelled L-serine was used as described above for the preparation of labelled L-glyceric acid.

The hydroxy[3-^{14}C]pyruvate was reduced to D-glyceric acid by reduced diphosphopyridine nucleotide (DPNH) and the specific dehydrogenase was obtained from parsley leaves as described by Stafford *et al.* (1954) [cf. Holzer & Holldorf (1957*b*)]. Hydroxy[3-^{14}C]pyruvate (156 μmoles) was incubated at 20° in a total volume of 50 ml. containing 1 ml. of D-glyceric acid dehydrogenase, 0·5 ml. of alcohol dehydrogenase, 10 mg. of diphosphopyridine nucleotide (DPN), 0·1 ml. of 2N-NH_3 soln. (to give pH 8·0), and 1 ml. of ethanol. After 30 min., all hydroxypyruvate was reduced as shown by the naphtharesorcinol test (Dickens & Williamson, 1958*b*). The mixture was heated for 10 min. in a boiling-water bath, cooled, filtered, and acidified with 2 ml. of 2N-H_2SO_4. After continuous extraction with ether for 4 days, 10 ml. of water was added to the extract, the ether was evaporated, and the residual aqueous layer contained D-[3-^{14}C]glyceric acid (115 μmoles, 5·0 μc). Chromatography on paper in ethanol–NH_3 soln.–water (8:1:1, by vol.) showed a single peak of radioactivity coinciding with glyceric acid similarly chromatographed (the unlabelled material was detected by spraying the dried paper with ethanolic chlorophenol red, 0·04%).

Other samples of D-[3-^{14}C]glyceric acid and unlabelled D-glyceric acid were similarly prepared enzymically from the corresponding lithium hydroxypyruvates, prepared according to Dickens & Williamson (1958*a*).

Action of D-*glyceric acid kinase on preparations of* D- *and* L-*glycerate*

Ichihara & Greenberg (1957) and Holzer & Holldorf (1957*a*) have described the preparation from liver of a kinase which specifically phosphorylates D-glyceric acid in the presence of adenosine triphosphate (ATP). The rat-liver enzyme has been used to assess the optical purity of the specimens of D- and L-glycerate prepared as above. Preliminary manometric experiments in bicarbonate media having shown that CO_2 evolution with ATP and the enzyme (plus magnesium and fluoride ions) occurred only

Table 1. *Action of* D-*glyceric acid kinase on samples of* D- *and* L-[3-^{14}C]*glycerate*

For details see text.

Addition	Tubes A	B	C	D
ATP (10 μmoles)	−	+	−	+
DL-Glycerate (10 μmoles)	+	+	+	+
D-Glycerate kinase (0·5 ml.)	+	+	+	+
[3-^{14}C]Glycerate (2 μmoles)	D-	D-	L-	L-
	After incubation			
Wt. of formaldehyde–dimedone (mg.)	36·3	36·4	37·7	37·9
Radioactivity (counts/min.)	191	8	607	605
[^{14}C]Glycerate utilized (%)	0	96	0	<0·5

with the D-isomer, the binding of the $CH_2 \cdot OH$ group in the [3-^{14}C]glycerate samples was determined by the loss of ability to liberate formaldehyde on treatment with periodate that occurs on phosphorylation of this group to give 3-phosphoglycerate. For this purpose tubes were set up as shown in Table 1. Potassium phosphate buffer, pH 7·4 (0·5 ml., 0·1 M), $MgCl_2$ (0·1 ml., 0·1 M) and water to a total volume of 2 ml. were added to each tube. Incubation for 40 min. at 37° was ended by cooling to 0°, addition of 0·5 ml. of 20% (w/v) trichloroacetic acid and removal of precipitated protein. Samples (1 ml.) were diluted to 25 ml. and portions of 1 ml. were neutralized with 2N-NaOH to phenol red; 5 ml. of water and 0·5 ml. of 0·2M-sodium periodate were added, followed after 10 min. at room temperature by 1·0 ml. of 5·4% (w/v) sodium arsenate. The solution was adjusted to approx. pH 3 and after a further 5 min. the volume was made up to 25 ml. After the addition of 1·0 ml. of 0·1 M-unlabelled formaldehyde to 5 ml. portions, the addition of 50 ml. of cold saturated aqueous dimedone precipitated the formaldehyde–dimedone which was filtered off on 1 cm.^{2} paper disks, washed, dried and counted on planchets as usual, with the results shown in Table 1. It is clear that only the D-form of glyceric acid is phosphorylated by the kinase and that the optical purity of the two samples is probably 96% or more, according to this sensitive assay method. Control experiments with DL-glycerate showed no interference by the presence of the L-isomer.

RESULTS

Metabolism of D- *and* L-*glycerates by rat-liver slices*

Table 2 summarizes the results of experiments in which [3-^{14}C]glycerate, D- and L-isomers, was incubated with rat-liver slices aerobically for 90 min. at 37° as described in the Methods section. In the first three experiments of Table 2 duplicate batches of slices from the same rat's liver were directly compared. Consistent differences were not observed, whether the D- or L-glycerate was the substrate. The percentage utilization, that is the difference between the total added periodate-liberated radioactivity and that remaining after incubation, was on the average 30%. Only a little over 3% of the added ^{14}C appeared as respiratory carbon dioxide, whereas about 10% entered the glucose molecule. This average glucose incorporation is a little lower than the mean value (15·7%) observed with hydroxy[3-^{14}C]pyruvate in otherwise similar experiments (Dickens & Williamson, 1959). In the case of rat no. 3, a parallel experiment in which 0·12 μC (15 μmoles) of lithium hydroxy[3-^{14}C]pyruvate was incubated with slices from the same liver, showed 12·5% of the ^{14}C as respiratory carbon dioxide, 18·7% incorporation into glucose, and 5·3% into serine.

In a few experiments in which the incorporation into serine of ^{14}C from the labelled glyceric acids was measured, the amount was also lower than for hydroxypyruvate, namely about 0·9–1·6% of the total added radioactivity. However, the observed incorporation into glucose of the labelled glycerate was rather variable and in some experiments reached values (e.g. rat no. 3, 16%) equal to those previously reported for the incorporation of hydroxy[3-^{14}C]pyruvate.

In order to decrease the possibility that the almost equal incorporation of ^{14}C from the samples of D- and L-glycerate might have been due to the highly preferential use of a radioactive impurity (e.g. of a trace of the opposite optical isomer), the experiments summarized in Table 3 were carried out. Graded amounts, down to one-tenth of the quantity of total glycerate normally used, were incubated with liver slices in the usual way.

Table 2. *Metabolism of* D- *and* L-[3-^{14}C]*glycerate in rat-liver slices*

Figures represent percentage of radioactivity present in the named fraction after incubation. For details see text.

	D-[3-^{14}C]Glycerate				L-[3-^{14}C]Glycerate			
			Incorporated into glucose				Incorporated into glucose	
Rat no.	$^{14}CO_2$*	Residual in medium†	% of added*	% of utilized‡	$^{14}CO_2$*	Residual in medium†	% of added*	% of utilized‡
1	2·6	61	7·2	18	3·4	68	7·9	25
	3·1	59	8·3	20	—	—	—	—
2	3·4	61	11·6	30	3·5	70	10·0	33
	4·3	67	11·4	34	—	—	—	—
3	3·0	80	15·4	77	4·4	78	12·9	58
	2·9	75	16·8	67	—	—	—	—
4	—	—	—	—	2·8	72	7·2	26
	—	—	—	—	3·1	76	6·3	26
Mean	3·2	67	11·8	41	3·4	73	8·9	34

* As percentage of total added radioactivity.

† By periodate oxidation and determination of radioactivity of liberated [^{14}C]formaldehyde; as percentage of added radioactivity.

‡ Calculated as percentage of (100-residual in medium).

Even at the concentration of 1 μmole/vessel there was no change in the percentage utilization, yield of radioactive carbon dioxide, or of glucose (Table 3).

The glucose formed in the experiments recorded in Table 2 was purified and degraded in order to determine the localization of ^{14}C in the various glucose carbon atoms (Table 4). Degradation with *L. casei* showed that 85–90% of the total radioactivity of the glucose resided in C-1 and C-6, with very low amounts in C-2 and C-5 and less than 10% in C-3 and C-4 combined. This picture was identical for D- and L-[3-^{14}C]glycerate as substrates. By purification of the glucose through the glucosazone and chemical degradation of the latter by means of periodate oxidation, C-6 was shown to have about 60% of the total glucose activity, whence it follows, by comparison with the figures for C-1 + C-6 in Table 4, that C-1 has about 30% of the total activity. Again, C-4 and C-5 together had less than 10%. Once more, no essential difference was observed whether D- or L-[3-^{14}C]-glycerate was the substrate used.

DISCUSSION

The results presented above leave little doubt that both D- and L-[3-^{14}C]glycerate are metabolized by rat-liver slices in a quantitatively closely similar manner. Both isomers are utilized to almost the same extent by liver slices; both make a similar, rather low, contribution to the respiratory carbon dioxide; both are incorporated into glucose formed by the slices to a similar extent and contribute ^{14}C nearly identically to the individual carbon atoms of the glucose molecule.

Enzymes metabolizing D- *and* L-*glycerate*

Since D-glycerate is the only isomer as yet reported to be present, as phosphoglycerate, in animal tissues, and a kinase for only the D-isomer is present in liver tissue (Ichihara & Greenberg, 1957; Holzer & Holldorf, 1957*a*); this result is a surprising one. However, it is worth noting that L-glycerate is a substrate, as effective as L-lactate, for the crystalline L-lactic dehydrogenase of animal

Table 3. *Effect of diminishing amount of added* D- *and* L-[3-^{14}C]*glycerate on the extent of its metabolism by rat-liver slices*

Expts. were under the usual incubation conditions (for details see text). Values represent percentage of total radioactivity in the fractions named. Specific activities were: D-isomer 0·05 μc/μmole and L-isomer 0·089 μc/μmole. Different rats were used for each of the two isomers.

Substrate ...	D-[3-^{14}C]Glycerate			L-[3-^{14}C]Glycerate		
Amount per vessel (μmoles)	CO_2	Residual in medium	Glucose	CO_2	Residual in medium	Glucose
10	3·7	51	8·6	2·5	61	3·5
4	—	—	—	2·5	62	3·8
2	5·8	49	9·7	2·2	56	3·3
1	4·8	48	7·3	—	—	—

Table 4. *Degradation of* [^{14}C]*glucose formed by liver slices incubated with* D- *or* L-[3-^{14}C]*glycerate*

(*a*) Degradation with *Lactobacillus casei*

Substrate	Radioactivity in glucose carbon atoms (% of total) C-1 + C-6	C-2 + C-5	C-3 + C-4
D-[3-^{14}C]Glycerate	90	1·5	9
L-[3-^{14}C]Glycerate	85	6	9·5

(*b*) Degradation of the glucosazones with periodate

	C-1 + C-2 + C-3	C-4 + C-5	C-6
D-[3-^{14}C]Glycerate*	39	3	58
	31	5	64
L-[3-^{14}C]Glycerate*	37	9	54
	28	5	67

* The upper and lower rows of figures in each bracketed set each refer to duplicate batches of liver slices from the same rat.

skeletal and heart muscle (Meister, 1952; Stafford *et al.* 1954) and is also a substrate for the flavocytochrome b_2 enzyme known as yeast lactic dehydrogenase (Dickens & Williamson, 1956), the product in the direction of dehydrogenation being hydroxypyruvate with both enzymes. We later found (Dickens & Williamson, 1958*a*, legend to Fig. 2) that a highly purified preparation of cytochrome b_2 kindly supplied by Professor E. Boeri is also active in this respect, like the cruder preparations used by Dickens & Williamson (1956). It remains to be seen whether the crystalline, yeast lactic-dehydrogenase preparation (Appleby & Morton, 1959*a*, *b*) also oxidizes L-glycerate. The identity of the product formed by dehydrogenation of L-glycerate with the yeast enzyme was originally shown to be hydroxypyruvate by colorimetric tests (Dickens & Williamson, 1956, 1958*b*) and later photometrically (F. Dickens & D. H. Williamson, unpublished work) by its reduction with DPNH and the dehydrogenase from parsley leaves (Stafford *et al.* 1954); this latter enzyme is specific for hydroxypyruvate and glyoxylate (cf. Holzer, 1959, p. 184). Thus yeast and animal tissues possess L-lactic-dehydrogenase systems which, though entirely different otherwise in their prosthetic group and coenzyme requirements, can both oxidize L-glycerate to hydroxypyruvate.

Many plant tissues contain, in addition to the above dehydrogenase which produces D-glycerate specifically from hydroxypyruvate and DPNH but does not reduce pyruvate to lactate, a second dehydrogenase system of the more familiar lactic-dehydrogenase type which reduces both pyruvate and hydroxypyruvate in presence of DPNH (Stafford *et al.* 1954). Presumably the product of the latter reaction would be L-glycerate, corresponding with the known presence of L-lactic dehydrogenase in plant tissues. Bacterial systems oxidizing both D- and L-lactate are also known (cf. De Ley & Schel, 1959).

Thus enzyme systems which dehydrogenate D- or L-lactate may or may not also dehydrogenate D- or L-glycerate, though more commonly the substrate specificity of systems, oxidizing α-hydroxy acids, is not marked. This applies also to L-malic dehydrogenase, which seems better described as an α-hydroxydicarboxylic acid dehydrogenase, according to Davies & Kun (1957).

In addition to the well-known DPN-linked L-lactic-dehydrogenase system of animal tissues, less well characterized enzymic systems which specifically oxidize D-lactate are present. In view of the low specificity of this type of enzyme for individual hydroxy acids (though specificity for the D- or L-series is usually complete) it seems desirable briefly to consider some examples here, although most of these have not yet been tested with D- and L-glycerate as substrates. Meyerhof & Lohmann (1926) found that rat-kidney slices and yeast cells could oxidize both D- and L-lactate. Baker (1952) found that washed particles from rat kidney and liver oxidized both D- and L-hydroxy acids, the activity towards the D-isomers being predominantly in the particles of the kidney preparation used; glycerate was not tested. With rabbit kidney and liver particles, D-lactate oxidation did not require added DPN and pyruvate was the product (Huennekens, Mahler & Nordmann, 1951; Mahler, Tomisek & Huennekens, 1953). Oxidation of D-lactate is not coupled with reductive carbohydrate synthesis in liver (Hoberman, 1958, 1960). Recently Tubbs & Greville (1959) have obtained a D-lactate dehydrogenase in soluble form from rabbit-kidney mitochondria; L-lactate was not attacked and the extracted enzyme required activation by aging (5 days at 0°) or by dilute cyanide.

The enzyme is also present in rat-liver mitochondria and is quite strongly active when tested with DL-glycerate as substrate, the product reacting as hydroxypyruvate in the Dickens & Williamson (1958*b*) naphtharesorcinol test (P. K. Tubbs & G. D. Greville, personal communication). The peculiar activation phenomenon with this enzyme may well have masked its presence in earlier attempts to isolate D-hydroxy acid dehydrogenases from animal tissues. The nature of the terminal acceptor and the physiological role of this enzyme remain to be investigated, but it appears possible that it may be a route for the reversible dehydrogenation of D-glycerate to hydroxypyruvate in animal tissues. The known DPN-linked L-lactate dehydrogenases of animal tissues constitute an electrophoretically heterogeneous group of proteins even from the same organ (Wieland & Pfleiderer, 1957; Wieland & Jeckel, 1957, see Holzer, 1959), but as a group would appear to be, like the crystalline L-lactic dehydrogenase of muscle, a probable source of reversible hydroxypyruvate production from L-glycerate in animal tissues. We have, in fact, found that incubation of hydroxypyruvate with mouse-ascites-tumour cells leads to the appearance of L-glycerate ($[\alpha]_D$, molybdate-enhanced, $-90°$ as sodium salt) in the medium (F. Dickens & D. H. Williamson, unpublished work).

Metabolism of D- *and* L-*glycerate in liver tissue*

The results described in the present paper show not only the approximately equal utilization of D- and L-glycerate by liver tissue of the rat, but also that the pattern of incorporation of [3-^{14}C] of both isomers into the glucose molecule by liver tissue is closely similar. In isotopic experiments of this kind it is important to eliminate the possibility

that the presence of a small percentage of the labelled opposite isomer might be responsible for the observed incorporation of isotope. It is believed that this explanation of the results may be excluded on the following grounds. The optical rotation of non-isotopic glycerate prepared by the same method as that used for the labelled material was satisfactory. The specific D-glycerate kinase, prepared from rat liver, phosphorylated the $^{14}CH_2 \cdot OH$ group of the D-[3-^{14}C]glycerate preparation (95 % of calculated amount) but not that of the L-[3-^{14}C]glycerate (< 0·5 %). The incubation of liver slices with progressively lower amounts of each labelled isomer did not affect the per cent of substrate utilized, the $^{14}CO_2$ liberated, or the incorporation of isotope into glucose by the liver slices (Table 3). Since the incorporation into glucose reached 16 % of that added in some experiments (Table 2), a large contamination with the opposite isomer would have been necessary to explain the results on this ground.

The actual distribution of radioactivity among the glucose carbon atoms (Table 4) showed approximately 60 % in C-6 and 30 % in C-1 of the glucose molecule, with much lower amounts in the other carbon atoms. A symmetrical entry of two molecules of [3-^{14}C]glycerate would, of course, be expected to give 50 % of the total activity of the glucose in each of the carbon atoms C-1 and C-6. The results therefore suggest that although the three-carbon chain of both D- and L-glycerate entered intact into glucose, with the unlabelled carboxyl groups as expected in C-3 and C-4, some dilution of the dihydroxyacetone phosphate which forms C-1 to C-3 of the glucose had occurred. Minor secondary reactions (probably $^{14}CO_2$ fixation into C-3 + C-4, accounting for about 9 % of the total incorporation) also occurred, as would be expected from the observed production of respiratory $^{14}CO_2$ (Tables 2 and 4).

At present, an explanation of these results for D-glycerate can be advanced but that for L-glycerate is more speculative. D-[3-^{14}C]Glycerate would be expected to be phosphorylated by the known D-glycerate kinase of liver to give 3-phospho-D-glycerate, which by the well-recognized reversal of D-glyceraldehyde 3-phosphate dehydrogenase would yield D-[3-^{14}C]glyceraldehyde 3-phosphate (cf. Holzer, 1959, p. 192). In the livers of well-fed rats, such as were used in our experiments, a pool of unlabelled dihydroxyacetone phosphate would be available which, through the action of aldolase, would form D-[6-^{14}C]fructose 1:6-diphosphate with the D-[3-^{14}C]glyceraldehyde 3-phosphate thus provided. At the same time, the highly active triose phosphate isomerase of rat liver would convert some of the labelled glyceraldehyde 3-phosphate into [1-^{14}C]dihydroxyacetone 1-phosphate. The aldolase condensation of the latter with D-[3-^{14}C]glyceraldehyde 3-phosphate would yield, as expected, D-[1:6-$^{14}C_2$]fructose 1:6-diphosphate. On this view the dilution of labelling at C-1 of the glucose formed via the fructose diphosphate is readily understandable, and this should be greater in fed animals. There is considerable evidence (see Wood, 1955) that equilibration by triose phosphate isomerase may be incomplete in such instances.

Although this explanation of D-glycerate metabolism is acceptable, that for L-glycerate requires consideration; and since both isomers are so similarly utilized, the fact that L-glycerate is not known to be capable of phosphorylation in liver, and in any event belongs to the wrong optical species for D-glucose formation, may even shed some doubt on the validity of the above explanation for D-glycerate. Naturally, the assumption of the presence in liver of an active racemase for glycerate would be an easy way out of this dilemma, but this assumption has no supporting evidence. On the other hand, both D- and L-glycerate, as we have seen, can be oxidized by liver enzyme systems to give hydroxypyruvate. [3-^{14}C]Hydroxypyruvate is very readily built into glucose by the liver (Dickens & Williamson, 1959), slice experiments closely comparable with those in the present work showing 15·7 ± 2·1 % incorporation, a value almost equal to the highest reported in this paper for [3-^{14}C]glycerate (Table 2). This raises the possibility that L-glycerate, and perhaps even D-glycerate also, may be metabolized via hydroxypyruvate, which as we have seen, could be formed enzymically from either isomer in liver. Until the route by which hydroxypyruvate is incorporated into the glucose molecule is known [for a discussion, see Dickens & Williamson (1959)], this possibility must remain speculative. At the present time, however, no suitable primary enzymic reaction of L-glycerate, other than its dehydrogenation to hydroxypyruvate, is known.

SUMMARY

1. The preparation and tests of optical purity of samples of D- and L-[3-^{14}C]glycerate are described.

2. The metabolism of these substances by respiring slices of rat liver has been studied.

3. Both isomers are consumed to an equal extent and contribute nearly identically to the ^{14}C appearing in the respiratory carbon dioxide and in the glucose formed. The latter may incorporate in 90 min. up to 16 % of the added isotope.

4. Distribution of ^{14}C from both isomers among the glucose carbon atoms is the same: approximately 60 % of the total labelling being in C-6,

30% in C-1, and 10% in C-3+C-4, of the glucose molecule.

5. Possible metabolic routes which might explain this unexpected metabolic similarity of the two isomers are discussed.

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The Reduction of Oxidized Glutathione in Erythrocyte Haemolysates in Pernicious Anaemia

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The erythrocytes and cells from certain tissues of vitamin B_{12}-deficient rats (Ling & Chow, 1953, 1954; Register, 1954; Kasbekar, Lavate, Rege & Sreenivasan, 1959) and chicks (Hsu, Chow & Okuda, 1959) but not mice (Jaffé, 1958) have unusually low concentrations of reduced glutathione, which rise to within the normal ranges soon after adequate administration of the vitamin. The reason for these low concentrations, however, has not been determined. Stekol (1954) found, using labelled glycine or cystine, that the synthesis of glutathione, *in vivo*, from its three amino acid components was not significantly different from the normal in the liver of vitamin B_{12}-deficient rats, Kasbekar & Sreenivasan (1959); who found a small decrease in the rate of this synthesis in erythrocytes taken from vitamin B_{12}-deficient rats, could not rule out that this might have been due to an observed slight haemolysis of the incubated cells.

Vitamin B_{12} has been implicated in the reduction of disulphides in rat-liver homogenates (Dubnoff, 1950), and it is therefore possible that the reduction of oxidized glutathione (a disulphide) is impaired

as a result of a deficiency of the vitamin. Such an impairment would probably entail low concentrations of reduced glutathione together with correspondingly high concentrations of oxidized glutathione. In addition, a deficiency in one of the components of the system reducing oxidized glutathione might be observed. This system, which involves glutathione reductase and triphosphopyridine nucleotide together with the corresponding dehydrogenases, catalyses the transfer of hydrogen from oxidizable substrates to oxidized glutathione (Rall & Lehninger, 1952; Conn & ennesland, 1951; Francoeur & Denstedt, 1954).

To test this theory, the concentrations of reduced and oxidized glutathione, triphosphopyridine nucleotide and oxidizable substrates have been measured in haemolysates of erythrocytes from patients with pernicious anaemia, before and after treatment with vitamin B_{12}, and compared with the corresponding values from normal erythrocytes. The outcome of this investigation is reported in this paper, a brief account of which has already been given (Jocelyn, 1959).

EXPERIMENTAL

Materials. Solutions of oxidized triphosphopyridine nucleotide (TPN), oxidized glutathione (GSSG) (both obtained from Boehringer und Sohne, Mannheim) and glucose 6-phosphate (British Drug Houses Ltd., Poole) (dried *in vacuo* before use) were made up immediately before the experiments. Disodium ethylenediaminetetra-acetate (EDTA) and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) were also from British Drug Houses Ltd. and reduced glutathione (GSH) was a gift from The Distillers' Co., Speke, Liverpool.

Blood from hospital patients with pernicious anaemia was kindly made available by Dr R. H. Girdwood, Department of Medicine, University of Edinburgh. Diagnosis had been confirmed by sternal puncture and blood-film examination. Figures for the red-cell counts were obtained from the Haematology Laboratory, Royal Infirmary, Edinburgh.

Preparation of the haemolysates. Packed human erythrocytes were obtained by centrifuging fresh oxalated blood for 10 min. at 1000 *g* and removing the plasma and buffy coat. They were washed once by suspending in an equal volume of 0·9% sodium chloride solution and recentrifuging. The separated erythrocytes were haemolysed with 9 vol. of oxygen-free water and the haemolysates used immediately.

Estimation of reduced glutathione. To the haemolysates (1–3 ml.), after incubation with the required additions, was added 5% metaphosphoric acid (0·5 ml.) to precipitate the proteins. After centrifuging, the clear, colourless, supernatant solution (3 ml.) was used for estimating GSH by the nitroprusside method (Grunerts & Phillips, 1951). All estimations were carried out in duplicate. The standard deviation of the readings from the mean for 20 estimations was $\pm 6{\cdot}5\,\mu$moles/100 ml. When haemolysates (3 ml.) containing added GSH (up to 0·8 μmole), EDTA (2 μmoles) and water to 4·5 ml. were incubated for up to 2 hr. at 37°, recoveries of the GSH averaged 95%. EDTA was included in all the experiments since its omission gave erratic results. There was no difference between the GSH content of haemolysates estimated at once or after incubation at 37° for 2 hr. The addition of GSSG (0·5 μmole) before this incubation did not affect the values obtained (see Pirie, 1959). GSH formed in haemolysates during incubation with the additions described was determined by subtracting from the GSH content of these mixtures that of the unincubated haemolysates alone.

Estimation of glutathione-reductase activity. The method of Collier & McRae (1955) was modified as follows: to the 1:10 haemolysate (1 ml.) were added GSSG (0·5 μmole), glucose 6-phosphate (0·5 μmole), TPN (0·1 μmole), EDTA

Table 1. *Recovery of oxidized glutathione added to haemolysates*

Mixtures of 1:10 haemolysate (3 ml.), glucose 6-phosphate (0·5 μmole), TPN (0·1 μmole), EDTA (2 μmoles), 0·1 M-tris–HCl solution, pH 7·4 (0·5 ml.) and GSSG as indicated in a final volume of 4·5 ml. were incubated at 37°. After 30 min. the proteins were precipitated and the GSH formed during the incubation estimated. The amount formed when no GSSG had been added to the mixture (0·02 μmole) was subtracted and the figures then obtained halved to give values for GSSG. Each recovery figure given represents the mean from duplicate incubations with the same haemolysate.

GSSG (μmole)	
Added	Found
0·05	0·09
0·10	0·11
0·15	0·15
0·20	0·20
0·25	0·24
0·30	0·30
0·35	0·35
0·40	0·38
0·45	0·43
0·50	0·50

Table 2. *Recovery of glucose 6-phosphate added to haemolysates*

Mixtures of 1:10 haemolysate (1 ml.), GSSG (0·5 μmole), TPN (0·1 μmole), EDTA (2 μmoles), 0·12 M-tris–HCl solution, pH 7·4 (0·5 ml.) and glucose 6-phosphate as indicated, to a final volume of 4·5 ml. were incubated at 37°. After 30 min. the proteins were precipitated and the GSH formed was estimated. GSH formed without added glucose 6-phosphate (0·04 μmole) was subtracted. The resultant figures were halved to give the stated recoveries which were the mean from duplicate incubations with the same haemolysate.

Glucose 6-phosphate (μmoles)	
Added	Found
0·05	0·04
0·10	0·10
0·15	0·145
0·20	0·18
0·25	0·235

(2 μmoles) and 0·12 M-tris–HCl solution, pH 7·4 (0·5 ml.) to a final volume of 4·5 ml. The mixture was incubated at 37° for 6 min., the proteins were precipitated and the GSH formed was estimated. This amount was used as the measure of glutathione-reductase activity since it varied linearly with changes in the volume of haemolysate used.

Estimation of oxidized glutathione. When amounts of GSSG up to 0·5 μmole were added to haemolysates together with glucose 6-phosphate and TPN, a quantitative conversion into GSH occurred within 30 min. (Table 1). Consequently, any GSH formed when GSSG was omitted represented the original concentration of GSSG in the haemolysates, thus providing a simple method for measuring the concentration in erythrocytes.

Estimation of triphosphopyridine nucleotide-reducing substrates. If TPN and excess of GSSG were both added to haemolysates, the amount of reduction which could occur was then limited by the concentration of glucose 6-phosphate also added. As shown in Table 2, the GSH formed after 30 min., with varying additions of glucose 6-phosphate, was 90–100% of the amount expected theoretically if 1 mole was required to reduce 1 mole of GSSG. Any GSH formed when no glucose 6-phosphate was added therefore represented the concentration of this substance or of other TPN-reducing substrates originally present in the haemolysate.

Estimation of total triphosphopyridine nucleotide. When GSSG and glucose 6-phosphate, but no TPN, were added to haemolysates, a slow reduction of the GSSG still occurred owing to the total (oxidized and reduced) TPN content of the haemolysate. To determine its concentration, the amount of GSH formed after 15 min. was compared with the amount formed in the same time when very small quantities of TPN (1–7 μm-moles) were added. Under these conditions the additional GSH formed was proportional to the concentration of added TPN up to 5 μm-moles (Fig. 1). The addition of diphosphopyridine nucleotide (up to 0·1 μmole) had no effect on the figures obtained. The TPN content of the original haemolysate was therefore found by extrapolation as shown for three different haemolysates in Fig. 1.

An assay method, with glutathione reductase to estimate total TPN, has been described (Anderson, 1953), but not previously applied to erythrocytes.

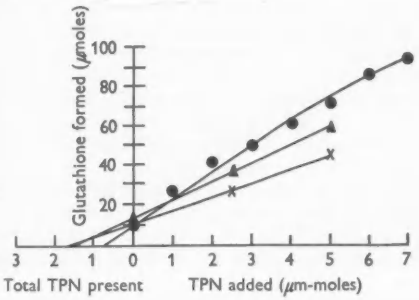

Fig. 1. Effect of added TPN on the reduction of GSSG in erythrocyte haemolysates. To 1 ml. of three different haemolysates (×, ▲, ●) was added GSSG (0·5 μmole), glucose 6-phosphate (0·5 μmole), EDTA (2 μmoles), TPN as indicated and 0·12 M-tris–HCl solution, pH 7·4 (0·5 ml.), to a final volume of 4·5 ml. The mixture was incubated for 15 min. at 37°, the proteins were precipitated and the GSH formed was estimated. The total TPN originally present in the haemolysates was found by extrapolation as shown.

RESULTS

With the methods described, glutathione-reductase activity and the concentration of GSH, GSSG, total TPN and TPN-reducing substrates have all been estimated on the same haemolysates. A range of the values found in haemolysates from the erythrocytes of ten normal human subjects is given in Table 3. Apart from the values for GSSG, these ranges are in good agreement with those found by other workers (Collier & McRae, 1955; Levitas, Robinson, Rosen, Huff & Perlzweig, 1947; Schlenk, 1942; see Jocelyn, 1958). The same estimations have also been performed on the haemolysates of erythrocytes from seven patients with pernicious anaemia both before and after treatment with vitamin B_{12}. The results are tabulated in Table 4 together with the red-cell count before treatment.

There are no significant departures ($> 2 \times$ S.D.) from the normal range of the values found in all seven untreated cases for GSH, glutathione-reductase activity and (in six of the cases) for total TPN. Also the activity of the enzyme is not affected by the addition of vitamin B_{12} to the haemolysate.

On the other hand, in three of the cases before treatment with vitamin B_{12}, the values obtained for GSSG are significantly above the normal range ($P = 0{\cdot}05$; $< 0{\cdot}01$; $< 0{\cdot}01$) and in the same three untreated cases an abnormally high level ($P < 0{\cdot}01$) of TPN-reducing substrates is also evident. Soon after administration of the vitamin (3–7 days) the two cases which could be followed show a large drop in the concentrations of TPN-reducing substrates and GSSG so that both are within the normal range. The losses in GSSG are accompanied

Table 3. *Range of values found in haemolysates of erythrocytes from ten normal subjects*

Glutathione-reductase activity is expressed as glutathione formed in 6 min. from 1 ml. of 1:10 haemolysate under the conditions given in Fig. 1. The other values are for 100 ml. of packed cells.

Substance	Concn. (μmoles) Range	Mean ± S.D.
GSH	160–300	220 ± 41
GSSG	0–15	5 ± 6·3
TPN-reducing substrates	14–40	25 ± 8·5
Total TPN	0·5–1·7	1·1 ± 0·4
Glutathione reductase	0·29–0·50	0·37 ± 0·08

Table 4. *Range of values found in haemolysates of erythrocytes from subjects with pernicious anaemia before and after treatment with vitamin B_{12}*

The dose of vitamin B_{12} injected in each case was 1 mg. Red-cell counts given are values before treatment. Other values are given as μmoles/100 ml. of packed cells except for glutathione-reductase activity which is expressed as GSH (μmoles) formed in 6 min. from 1 ml. of 1:10 haemolysate; values in brackets are the corresponding activities found when vitamin B_{12} was added to the haemolysate before estimation at a concentration of 0·1 μg./ml. Other values were estimated in duplicate and, except for TPN, both results are given. These are based on the mean of the two readings shown for GSH.

Subject	Time of venepuncture (before or after dose of vitamin B_{12})	Red-cell count (millions)	GSH	GSSG	Glutathione-reductase activity	Total TPN	TPN-reducing substrates
1	Before	1·8	223, 237	53·5, 56·5	27 (28)	0·7	65, 70
	3 days after		323, 343	0·5, −0·5	28, 29	0·6	10, 15
2	Before	2·0	255, 267	28·5, 30·5	42 (38)	2·4	74, 72
	7 days after		301, 317	0·5, 7	—	—	18, 18
3	Before	1·39	186, 190	15, 7	—	—	—
	10 days after		200, 207	−1·5, 1	—	—	—
4	Before	0·9	270, 264	15, 18	34 (29)	1·2	55, 56
5	Before	—	262, 250	0, 8	35 (32)	1·1	10, 15
6	Before	2·5	150, 160	10, 2	38 (34)	1·25	30, 35
7	Before	1·76	238, 238	−0·5, 2·5	50 (51)	1·2	15, 20

by a gain in the concentration of GSH of approximately the amount expected if the lost GSSG had been reduced to GSH *in vivo*. In both cases the new concentration of GSH is significantly above the normal range.

DISCUSSION

The reduction of GSSG in haemolysates, under the conditions used, involves both glutathione reductase and glucose 6-phosphate dehydrogenase but since addition of the purified dehydrogenase does not increase the rate of the reduction, this is actually limited by the activity of glutathione reductase, of which it is therefore a convenient measure (Collier & McRae, 1955). The use of this glutathione-reductase system to estimate the erythrocyte concentration of one of its components, GSSG, glucose 6-phosphate (or TPN-reducing substrates) or total TPN merely involves omitting this component from the additions made, when its endogenous concentration limits the amount of GSH which can be formed or, for TPN, the rate of its formation.

Glutathione reductase has occasionally been used (e.g. Martin & McIlwain, 1959) to convert GSSG into GSH but the enzyme has usually been supplied from an exogenous source and used to reduce the GSSG present in a protein-free filtrate. In estimating GSSG in erythrocytes by using the reductase which they already contain, the reduction is carried out before precipitating the proteins. This offers an important advantage over other procedures used.

Thus it is uncertain whether (or to what extent) GSSG is formed during the precipitation of proteins from a solution containing GSH. Consequently, subsequent values found may overestimate the concentration of GSSG in the original solutions. However, if GSSG is reduced to GSH before precipitation of the proteins and then estimated from the difference between the GSH content of the protein-free filtrates from reduced and unreduced solutions, any oxidation occurring during the precipitation will be common to both solutions and cancel out. Thus though the GSH values found will be smaller by the amounts oxidized, those for GSSG will represent the true values present before precipitation. Applying this method to haemolysates of erythrocytes, the values obtained for GSSG are in fact considerably lower than those found in human blood or erythrocytes by other workers. Thus the mean value for GSSG (from ten normal subjects) expressed as GSH is 0–6 % of the total glutathione (GSH + GSSG) found whereas Bhattacharya, Robson & Stewart (1955) found values for blood GSSG of 10–25 % of total glutathione.

The GSH formed when glucose 6-phosphate is omitted from the glutathione reductase system probably represents mainly that arising through the presence of endogenous glucose 6-phosphate. The other chief substrate in erythrocytes involving a TPN-reducing dehydrogenase is 6-phosphogluconic acid. This substance is capable of reducing GSSG in haemolysates (Carson, Flanagan, Ickes & Alving, 1956) but does not seem to do so under the conditions used in the present work since, though

6-phosphogluconate arises from the dehydrogenation of glucose 6-phosphate, only 1 mole of GSSG is in fact reduced by the latter substance [cf. Conn & Vennesland (1951) who found a 13–25% further reduction from glucose 6-phosphate added to wheat-germ extract].

From the results for these estimations performed on haemolysates of erythrocytes both from normal subjects and from patients with pernicious anaemia, some points may be noted:

(1) The finding that, in untreated pernicious anaemia, the concentrations of GSH are within the normal range contrasts with the low concentrations found in erythrocytes from vitamin B_{12}-deficient animals (see introduction) and conflicts with the finding of Ling & Chow (1953) of a low concentration in the one case of pernicious anaemia they examined.

(2) These authors observed a rise in GSH, after treatment of their one case with vitamin B_{12}, similar to the rise observed in vitamin B_{12}-deficient animals after administering the vitamin. Thus the elevation of GSH noted in the present work in two of the cases after treatment with vitamin B_{12} would seem to be in accordance with these findings. This additional GSH seems to originate from a reduction of the abnormal amount of GSSG found in the untreated cases because, after treatment, this GSSG falls approximately to the extent that the GSH rises. Thus it is possible that the low concentration of GSH found in the vitamin B_{12}-deficient animals is also due to conversion into GSSG and that this process is reversed when the vitamin is given.

(3) In the three untreated cases where high concentrations of GSSG were found, the concentration of TPN-reducing substrates was also high. Since in the two cases examined after treatment both of these values had reverted to within the normal range, it seems likely that their elevation was due to a single deficiency correctable by vitamin B_{12}. A deficiency in glucose 6-phosphate-dehydrogenase activity, which would inhibit both the utilization of glucose 6-phosphate through the pentose phosphate pathway and also the reduction of GSSG, has been shown (Carson *et al.* 1956; Beutler, 1959) to account for the low concentrations of GSH found in erythrocytes of subjects with certain drug-sensitive haemolytic anaemias. Such a deficiency however, necessarily affects the activity of glutathione reductase when measured in haemolysates by coupling the two enzyme reactions, and in the present work glutathione-reductase activities were found to be normal and uninfluenced by vitamin B_{12}.

A block in the glycolytic pathway from glucose to lactate in erythrocytes would also be likely to lead to an accumulation of glucose 6-phosphate and this explanation would be in accord with the impaired tolerance to glucose found in vitamin B_{12}-deficient rats (Ling & Chow, 1954). Though such a block could not plausibly account for an accumulation of GSSG, there is the converse possibility that GSSG, present in excessive amount, might partly reversibly inactivate thiol-dependent enzymes of this pathway (e.g. glyceraldehyde 3-phosphate dehydrogenase) in the manner demonstrated by Hopkins, Morgan & Lutwak-Mann (1938). The high concentrations of TPN-reducing substrates found in the cases of untreated pernicious anaemia would then be a consequence of the high concentrations of GSSG.

The concentrations of GSSG probably reflect an intracellular equilibrium between its loss by reduction and its formation from GSH by oxidation. This would explain why some GSSG coexists with some TPN-reducing substrates even in normal subjects (Table 3). Since, despite the high concentrations found, the reduction of GSSG is unimpaired in untreated pernicious anaemia, the possibility arises that the rate of oxidation of GSH is increased above the normal in this disease and that this is the primary defect correctable by administration of vitamin B_{12}.

SUMMARY

1. The reduction of oxidized glutathione to the reduced form in erythrocyte haemolysates containing glucose 6-phosphate and oxidized triphosphopyridine nucleotide has been used as the basis for estimating glutathione-reductase activity and the concentrations of oxidized glutathione, triphosphopyridine nucleotide-reducible substrates and total triphosphopyridine nucleotide.

2. These estimations, together with that of reduced glutathione, have been performed on haemolysates from erythrocytes from ten normal human subjects and seven with pernicious anaemia.

3. In untreated pernicious anaemia, the concentrations of reduced glutathione, glutathione reductase and triphosphopyridine nucleotide were within the normal range whereas in three cases those of oxidized glutathione and triphosphopyridine nucleotide-reducing substrates were above it.

4. After treatment with vitamin B_{12}, the oxidized glutathione appeared to be converted into reduced glutathione, and the concentration of triphosphopyridine nucleotide-reducing substrates fell to within the normal range.

5. The significance of these findings is discussed and the suggestion made that vitamin B_{12} plays a part in inhibiting the oxidation of reduced glutathione.

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The Effect of some Protein Solutions on the Oxidation of Glutathione in Oxygenated Erythrocytes

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Though much is known about the oxidation *in vitro* of reduced glutathione (Barron, 1951; Isherwood, 1959), the corresponding intracellular reaction has been little studied. Meldrum (1932) aerated glucose-depleted ox erythrocytes and observed a slow fall in the concentration of glutathione which, since it was prevented by the addition of glucose, he attributed to its intracellular oxidation. Klebanoff (1957) using this system found that besides glucose, adenosine, inosine and 33% plasma (but not bovine-serum albumin) prevented the loss of glutathione by oxidation when added to the saline in which the cells were suspended. The effect of plasma was said to be due to its supplying 'utilizable metabolites to the cell'.

Enzymes have also been described which convert glutathione into its oxidized form with or without oxygen itself. Among these are glutathione peroxidase (Mills, 1957), cytochrome *c*–cytochrome *c* oxidase (Boeri, Baltscheffsky, Bonnichsen & Gustav-Paul, 1953), glutathione–homocystine transhydrogenase (Racker, 1955*b*) and dehydroascorbic acid reductase (Hopkins & Morgan, 1936). Apart from the last these have all been found in animal tissues but their quantitative importance as routes for the intracellular oxidation of glutathione is not known. However, in view of the speed of the metal-catalysed oxidation *in vitro* (see Isherwood, 1959) and the presence of such catalytic metals (e.g. iron and copper) in the tissues, it seems likely that the major proportion of glutathione oxidized intracellularly is oxidized by direct reaction with oxygen itself.

In the presence of glucose, net intracellular oxidation of glutathione cannot normally be observed (Meldrum, 1932; Klebanoff, 1957). This is probably due to the activity of the widely distributed enzyme, glutathione reductase which mediates the reduction of the oxidized glutathione by means of reduced triphosphopyridine nucleotide (Rall & Lehninger, 1952). Regeneration of the latter from its oxidized form requires the metabolism of glucose (see Horecker, 1951). To investigate systematically the intracellular oxidation of glutathione, it is therefore necessary to use cells depleted of glucose, thus enabling the oxidation to be followed, uncomplicated by any subsequent reduction of the oxidized glutathione formed.

This paper describes, with glucose-depleted human erythrocytes, how the rate of oxidation of glutathione in the oxygenated cells can vary according to the nature of the medium in which the cells are suspended; evidence is also presented to show the occurrence in human serum of a factor with an inhibitory effect on the oxidation.

EXPERIMENTAL

Materials. Oxidized glutathione (GSSG) was obtained from Boehringer und Söhne, Mannheim; reduced glutathione (GSH) was a gift from The Distillers Co. Ltd., Speke, Liverpool; disodium ethylenediaminetetra-acetate (EDTA) was from British Drug Houses Ltd., Poole; bovine-serum albumin, fraction V, and bovine γ-globulins, fraction II, were from Armour Biochemicals Ltd., Eastbourne; human-serum albumin, fraction V (HSA), was kindly provided by the Blood Transfusion Service, Royal Infirmary, Edinburgh. Both albumin fractions were prepared as described by Cohn *et al.* (1946).

Dialysis. Solutions were dialysed against running water at 16–18° in Visking $\frac{1}{32}$ in. cellophan tubing. Purified protein solutions were dialysed for 6–10 hr. before dilution to the required concentration. These solutions were kept at 5° and freshly made up each week.

Dialysed serum. This was prepared from human oxalated plasma. After dialysis for the desired time, NaCl (0·65 g./100 ml. of the dialysed solution) was added and dialysed plasma was converted into dialysed serum by incubating at 37° for 10 min., when a clot of fibrin formed and was squeezed from the clear solution.

Washed erythrocytes. Erythrocytes were obtained from fresh, oxalated human blood by centrifuging for 10 min. at 1000 *g* and removing the plasma and buffy coat. When pooled erythrocytes were used, the plasma was separated before mixing the cells. Erythrocytes were resuspended in 2 vol. of 0·9% sodium chloride containing EDTA (2 mM) and incubated at 37° for 15 min. The cells, separated from the sodium chloride–EDTA solution by centrifuging, were incubated for a further 15 min. with 2 vol. of fresh solution. The reseparated washed cells were then stored at 5°. After this treatment, glucose, estimated according to Folin & Wu (1920) could not be detected.

During washing, losses in GSH and GSSG (total glutathione) were often incurred (see also Martin & McIlwain, 1959). If EDTA was omitted from the 0·9% sodium chloride, these losses were much greater. Washed erythrocytes were used for the oxygenation experiments within 30 hr. of the shedding of the original blood.

Treatment of washed cells with carbon monoxide. Carbon monoxide was prepared by the action of concentrated sulphuric acid on formic acid and freed from acid fumes by bubbling through 10% sodium hydroxide solution. The gas was bubbled for 30 min. through a suspension of the washed erythrocytes in 1 vol. of sodium chloride–EDTA solution in the dark. The separated cells were kept in a stoppered container at 5°. In the experiments in which these erythrocytes were used, the corresponding ungassed cells (controls) were otherwise treated in exactly the same way.

Oxygenation of erythrocytes. Oxygen, saturated with water vapour at 23° or 37°, was bubbled through tubes containing suspensions of washed erythrocytes in the required solutions at a flow rate of about 5 ml./min. Foaming was prevented by addition of 1 drop of Silicone M.S. Antifoam emulsion 'RD' (Hopkin and Williams Ltd., Chadwell Heath, Essex). The outlet of one tube was connected to the inlet of another, up to 12 oxygenations thus being performed at the same time and with the same oxygen flow rate. Tubes were immersed in water at 23° or 37° and kept in an incubator at the same temperature. Samples of the oxygenated mixtures, pipetted from the tubes at the required intervals, were centrifuged for 10 min. at 1000 *g* and the supernatant solutions were separated from the packed erythrocytes.

Reduced glutathione and total glutathione. These were estimated on haemolysates of the erythrocytes as already described (Jocelyn, 1960). All duplicate readings agreed within 20 μmoles/100 ml. of cells. The standard deviation from the mean was 6·5 μmoles/100 ml. of cells.

Oxyhaemoglobin. A Brinkmann Haemoreflector (Kipp and Sons, Delft, Holland) was used. Measurements were made on a suspension of erythrocytes in the required solutions. Zero oxyhaemoglobin was taken as the reading with a sample of unoxygenated erythrocytes in 1 vol. of 0·9% sodium chloride solution containing sodium dithionite (1 g./100 ml.).

RESULTS

When intact human erythrocytes are oxygenated at 37°, a gradual fall in their GSH content occurs. The rate of this fall varies from one batch of cells to another and also according to the constitution of the medium in which the cells are suspended during the oxygenation. Two media which have been extensively used are 0·9% sodium chloride (saline) and a solution of human-serum albumin containing 0·75% sodium chloride to produce isosmotic conditions (HSA solution).

In forty-two experiments, each with a different batch of fresh, washed, pooled cells, the rates of fall of GSH in the same cells oxygenated at 37° in both these media have been noted together with the GSH content before oxygenation. The rate of fall of GSH in different cells varies between 5 and 50 μmoles of GSH/100 ml. of cells/hr. in saline and is 15–60 μmoles of GSH/hr. in HSA solution. The data for each batch of cells has been classified in one of three groups according to the order of the rate of fall in saline (Table 1). In each group, the range and mean have been included for the initial cell concentrations of GSH before oxygenation. Also included are the differences found between the GSH content of the cells oxygenated for 3 hr. in saline and the same cells oxygenated for 3 hr. in HSA solution. From this it is seen that the mean initial GSH concentration found in group 1 (with the lowest rates of fall) is significantly lower ($> 2 \times$ S.D.) than that found in group 3 (with the highest rates of fall).

The data in Table 1 also show that the mean fall of GSH in HSA solution after 3 hr. of oxygenation

Table 1. *A comparison of the rates of loss of reduced glutathione in different batches of human erythrocytes, oxygenated in saline or human-serum albumin solution with the corresponding initial concentrations of reduced glutathione*

Figures for the rate of loss of GSH/hr. are the average from the rate of loss over the first 3 hr. of oxygenation. A different batch of cells was used for each experiment. The initial concentration of GSH is the concentration found after washing but before oxygenation. The HSA solution contained 4 g. of HSA/100 ml. of 0·75% NaCl. The experiments were all carried out as described in Fig. 1. The values given for GSH are in μmoles/100 ml. of cells.

Group	No. of experiments	Rate of loss of GSH/hr. in		Difference after 3 hr. of oxygenation between GSH of cells in saline and the same cells in HSA solution		Initial GSH concn.	
		Saline	HSA solution	Range	Mean ± S.D.	Range	Mean ± S.D.
1	17	5–16	15–43	18–113	51 ± 29	100–270	178 ± 48
2	17	17–33	25–53	10–75	43·5 ± 17	140–275	222 ± 38
3	8	34–50	45–60	8–57	34·5 ± 15·5	220–300	256 ± 24

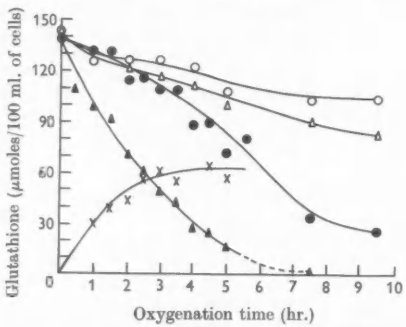

Fig. 1. Effect of oxygenation on the GSH and total glutathione content of washed human erythrocytes, oxygenated in 0·9% NaCl solution (GSH, ●; total, ○) or in HSA solution (GSH, ▲; total, △) and upon the difference in GSH (×) between cells from these two media. A mixture of 1 vol. of the washed pooled erythrocytes (18 ml.) and 1 vol. of 0·9% NaCl or 1 vol. of HSA solution containing 4 g. of HSA/100 ml. of 0·75% NaCl was oxygenated at 37°. At intervals, 2 ml. of the mixtures were withdrawn and glutathione (GSH and total) determined on the separated cells.

is greater by 34·5–51 μmoles of GSH/100 ml. of cells than the fall for the same cells oxygenated for the same time in saline. The different rates of oxidation in these two media are illustrated for a single experiment in Fig. 1, in which the GSH concentrations of the same batch of pooled cells, oxygenated in either saline or HSA solution, have been plotted against time of oxygenation. In both media, the rate of fall of GSH does not significantly slow up until the cell concentration of GSH has reached a low value. The difference between the GSH content of the cells oxygenated in saline and HSA solution for the same time has also been plotted against time. This difference increases to a maximum value after 3 hr. of oxygenation. The increase of this difference with time of oxygenation has also been followed in twenty-three of the experiments summarized in Table 1. In fifteen of them, a maximum difference was found after 3 hr. of oxygenation (as in Fig. 1) and in the other eight the difference was still diverging after this time (as in Fig. 5).

Different rates of fall of GSH in saline and HSA solution have also been observed with cells from single blood donors (see Fig. 3).

Effect of oxygenation on the concentration of total glutathione. To show whether the fall in the concentration of GSH in oxygenated cells can be accounted for by its oxidation to GSSG, the levels of GSH and total glutathione have been measured simultaneously at intervals during the oxygenation. The results of one experiment (Fig. 1) show that GSH is largely oxidized to GSSG but that there is also a slow fall in the concentration of total glutathione. This fall is greater for the cells oxygenated in HSA solution than for the cells oxygenated in saline. To determine the conditions under which this fall in total glutathione occurs, erythrocytes have been oxygenated in HSA solution containing increasing amounts of glucose (Table 2). Glucose at a concentration of 120 mg./100 ml. of HSA solution prevents the oxidation of GSH and maintains the level in the erythrocytes at the initial value throughout 3 hr. of oxygenation (see also Meldrum, 1932; Klebanoff, 1957). When the glucose concentration is decreased below this value, however, oxygenation of the cells then leads to a fall in the concentration of GSH and the fall is greater the smaller the concentration of glucose in the original solution. From the corresponding figures for the total glutathione in these cells the concentration of GSSG can be calculated. It is then evident that the losses observed in total glutathione during oxygenation increase in magnitude with increasing concentration of the GSSG found. After oxygenation for 3 hr., only 2–3% of the cells are found to be haemolysed and added

Table 2. *Effect of varying concentrations of glucose on the concentrations of reduced and oxidized glutathione in washed oxygenated human erythrocytes*

The washed pooled erythrocytes (1·5 ml.) were oxygenated for 3 hr. at 37° in 0·75% NaCl solution (1·5 ml.) containing HSA (4 g./100 ml.) and glucose as indicated. Percentage haemolysis after 3 hr. was determined as described by Klebanoff (1957) and reduced and total glutathione were estimated on haemolysates of the separated cells. Recoveries of GSSG added to the haemolysates are given as additional GSH formed/100 ml. of cells after the enzymic reduction. Loss in total glutathione is based on the content of the unincubated cells.

Glucose added (mg./100 ml. of HSA solution)	Glutathione (μmoles/100 ml. of cells)				Recovery of added GSSG (100% = 200)	Haemolysis (%)
	Reduced	Total	Oxidized	Total lost		
0	10	115	105	60	190	2·5
40	45	137	92	38	192	2
80	95	152	57	23	197	2
120	140	160	20	15	200	2
200	135	160	25	15	195	2·5
Unincubated cells	137	175	38	—	190	—

Table 3. *Effect of incubation on the concentration of total glutathione of previously oxygenated, washed, human erythrocytes*

Washed pooled erythrocytes, after oxygenation in 1 vol. of 0·9% NaCl at 37° for 11 hr., were incubated at 37° without oxygenation under the conditions shown. Samples were taken at the stated intervals for estimation of reduced or total glutathione.

Conditions	Glutathione (μmoles/100 ml. of cells)				
	Before incubation		After 1 hr. Total	After 3 hr. Total	After 5 hr. Total
	Reduced	Total			
Unoxygenated cells	190	220	—	—	—
Oxygenated cells alone	55	172	130	100	82
Oxygenated cells with 1 vol. of 0·9% NaCl	—	—	125	97	87
Oxygenated cells with 1 vol. of 0·75% NaCl containing HSA (4 g./100 ml.)	—	—	130	102	80

GSSG is still quantitatively recovered from the haemolysates (see Jocelyn, 1960).

The effect of incubating cells, previously oxygenated to produce a high concentration of GSSG, has also been examined (Table 3). The cells have been kept at 37° without further oxygenation and the concentrations of total glutathione determined at intervals over 5 hr. There is a steady fall in the concentration of total glutathione over this period and the rate of fall is unaffected whether the cells are incubated alone or suspended in either saline or HSA solutions.

Mechanism of the effect of albumin. The difference between the GSH concentration of the same cells oxygenated in saline and in HSA solution has been chiefly studied at a concentration of 4 g. of HSA/100 ml. of solution. Decreasing this amount to 0·6–0·8 g./100 ml., however, does not affect the GSH difference and below this value the difference declines roughly linearly with decreasing concentration of HSA (Fig. 2). This result has been obtained with five batches of cells and with periods of oxygenation varying between 2 and 4 hr. If the cells in saline or HSA solution are treated with nitrogen instead of oxygen, there is no fall in the GSH concentration in either medium (Fig. 2).

Fig. 2. Influence of HSA on the level of GSH in washed pooled human erythrocytes treated in HSA solutions with oxygen for 4 hr. (●) or 2·5 hr. (▲) or with nitrogen for 2·5 hr. (△). Values after 2·5 hr. were obtained with one batch of pooled cells and values after 4 hr. with another batch. Oxygen or nitrogen at the same flow rate was bubbled through the washed erythrocytes in 1 vol. of the solution at 37° for the times stated. GSH was estimated on the separated cells: the mean of duplicate readings is given for the 2·5 hr. values.

When cells are oxygenated with saline, Krebs–Ringer or Krebs–Ringer-phosphate solutions, made up as described by Umbreit, Burris & Stauffer (1949), there are no differences between the rates of oxidation of GSH in these solutions or between the amount of increase when HSA is added (Table 4). On the other hand, the rate of oxidation of GSH in cells oxygenated in either saline or HSA solution is considerably increased when cupric ions are added to the solutions before oxygenation.

A comparison of the effect at various concentrations of HSA and iodine-oxidized HSA on the rates of oxidation of GSH in oxygenated erythrocytes shows that there are no significant differences between the two substances (Table 5).

Is oxyhaemoglobin involved in the oxidation? Oxyhaemoglobin concentrations, measured by reflectance in the intact cells after oxygenation with HSA solutions, decrease with increasing concentration of HSA in the solution used (Table 6). To test whether the effect of albumin on the oxidation of GSH might involve oxyhaemoglobin, the rates of this oxidation in oxygenated cells pretreated with carbon monoxide have been compared with the rates for the same untreated cells. The results from three experiments in which both sets of cells have been oxygenated in saline and HSA solution are shown in Table 7. Treatment with carbon monoxide increases the rate of oxidation over that found with the untreated cells, in both solutions by a factor of 1·2–1·4, and there is consequently a similar increase in the difference between the GSH of cells oxygenated in the two media. Treatment of erythrocytes with carbon monoxide is known to increase the concentration of GSH well above that found for the corresponding untreated cells (Numata, 1940). This increase, measured before oxygenation, is to about 1·2–1·5 times the concentration in the untreated cells, i.e. about the same amount as the subsequent increase in the rate of oxidation. On oxygenating the carbon monoxide-treated cells, there is a decrease (30–35 %) in the measured percentage saturation with carbon monoxide which is complete after 2 hr. of oxygenation, nevertheless the rates of oxidation in the treated cells do not decrease during this period.

The effect of some other protein solutions on the oxidation. A comparison has been made between the rates of oxidation of GSH in the same cells oxygenated with saline, HSA solution, bovine-serum albumin solution and bovine γ-globulin solution, all present at the same concentration in 0·75 % sodium chloride. The results of four experiments are summarized in Table 8. In three experiments, the rate with bovine-serum albumin solution is not significantly different from the rate with

Table 4. *Influence of the composition of the saline medium, with or without human-serum albumin, on the oxidation of reduced glutathione in oxygenated washed human erythrocytes*

HSA concentrations were 4 g./100 ml. of the saline solution. Krebs–Ringer and Krebs–Ringer-phosphate solutions were made up as described by Umbreit, Burris & Stauffer (1949) but where HSA was added the 0·9 % NaCl prescribed was replaced by 0·75 % NaCl. The washed pooled erythrocytes were oxygenated with 1 vol. of the solution for 3 hr. at 37° and the cell GSH concentration subsequently estimated.

	GSH (μmoles/100 ml. of cells)	
Medium	Alone	With albumin
0·9 % NaCl	150	130
Krebs–Ringer solution	155	132
Krebs–Ringer-phosphate solution	157	130
0·9 % NaCl + $CuSO_4$ (0·016 mM)	110	92

Table 5. *Comparison of the effects of untreated and iodinated human-serum-albumin solutions on the oxidation of reduced glutathione in oxygenated washed human erythrocytes*

Iodinated HSA was prepared according to Hughes & Straessle (1950). The proteins were dissolved in 0·75 % NaCl to give the concentrations shown and the washed pooled erythrocytes oxygenated for 3 hr. at 37° with 1 vol. of these protein solutions. GSH was estimated on haemolysates of the separated cells.

Protein concentration (g./100 ml. of solution)	GSH (μmoles/100 ml. of cells) in	
	HSA solution	Iodinated HSA solution
0	120	120
0·8	96	90
1·6	90	87
2·4	84	88
3·2	90	90
4·0	88	92

Table 6. *Comparative oxyhaemoglobin concentrations of washed human erythrocytes oxygenated in human-serum-albumin solutions*

The HSA was dissolved in 0·75 % NaCl solution to give the concentrations shown. After oxygenation of the washed pooled erythrocytes at 37° for 1·5 hr. with 1 vol. of the solution, oxyhaemoglobin was measured as described in the text, the oxyhaemoglobin concentration of the cells oxygenated with 0·75 % NaCl alone being taken as 100.

HSA concentration (g./100 ml. of solution)	Oxyhaemoglobin
0	100
0·8	92
1·6	90
2·4	84
3·2	78
4·0	69

Table 7. *Comparison of the rates of oxidation of reduced glutathione in untreated and carbon monoxide-treated, oxygenated washed human erythrocytes*

HSA solution contained 4 g. of HSA/100 ml. of 0·75 % NaCl. Carbon monoxide-treated, and untreated, washed pooled erythrocytes were oxygenated at 37° in 1 vol. of the medium and GSH determined on the separated cells. Carbon monoxide (% saturation) was estimated as described by Gray & Sanford (1946).

Expt. no.	Medium	Glutathione (μmoles/100 ml. of cells)								Carbon monoxide saturation (%)		
		Oxidized/hr. (average over 3 hr.)			Difference after 4 hr. between cells oxygenated in saline and HSA solution		Unoxygenated washed cells			Before oxygenation	After 2 hr.	After 4 hr.
		Treated	Untreated	Ratio	Treated	Untreated	Treated	Untreated	Ratio			
1	Saline	22·5	16	1·4	55	45	210	140	1·5	—	—	—
	HSA solution	35	25	1·4	—	—	—	—	—	—	—	—
2	Saline	20	16	1·25	60	40	240	174	1·38	—	—	—
	HSA solution	37·5	27·5	1·36	—	—	—	—	—	—	—	—
3	Saline	55	45	1·22	30	20	310	265	1·16	86	56	56
	HSA solution	62·5	50	1·24	—	—	—	—	—	—	48	50

saline alone and in the fourth, the two rates do not greatly differ. On the other hand, in all four experiments the rate with bovine γ-globulin solution is much greater and equal to the rate with HSA solution.

Experiments with dialysed serum. Human oxalated plasma has been dialysed to remove glucose and the fibrinogen subsequently precipitated as fibrin. If washed cells are oxygenated in this dialysed solution containing added sodium chloride (dialysed serum), the rate of oxidation of glutathione in the cells is profoundly affected by the way in which the dialysed serum has been previously treated. If erythrocytes, separated from blood taken from a single donor, are oxygenated with dialysed serum (dialysis time, 24 hr.) prepared from plasma from the same blood, the rate of oxidation of GSH in the cells is the same as for saline (Fig. 3*A*). If however this dialysed serum has, before the oxygenation, been heated at 70° for 1 min., the rate is then greater and equal to the rate observed with HSA solution (Fig. 3*A*). When the unheated dialysed serum is redialysed (for a further 20 hr.) and then used again as the medium for oxygenating the same cells (now 24 hr. older), the rate of oxidation of GSH in these cells is much faster than with the original dialysed serum and is steeper even than that of the cells oxygenated at the same time in HSA solution (Fig. 3*B*). The same qualitative results are obtained 24 hr. later still, when fresh pooled erythrocytes are oxygenated with this same dialysed and redialysed serum simultaneously (Fig. 3*C*).

Essentially the same effects are also observed when dialysed serum, prepared from fresh pooled plasma, is used in place of that prepared from the blood of a single donor. The length of time for which this pooled dialysed serum has been dialysed also greatly affects the length of time during which it needs to be heated at 70° in order to increase the rate of oxidation of pooled erythrocytes oxygenated in it from the rate in saline to the rate in HSA solution (Table 9). After dialysis for 20–24 hr., only brief heating ($\frac{1}{2}$–3 min.) is required (see also Fig. 3*A*); with shorter dialysis (8 hr.), heating for 3–10 min. is necessary whereas after only 3 hr. of dialysis, the heating must be from 20 to 40 min. Moreover, with these shorter periods of dialysis, the longer the period of heating within the time limits stated, the nearer is the subsequently observed rate of oxidation of GSH in cells oxygenated in the heated dialysed serum to that found in HSA solution.

When the rate of oxidation of GSH found in cells oxygenated in dialysed serum follows or is near to the rate with saline, it is convenient to describe this batch of dialysed serum as 'active' and when the rate followed is at or greater than the rate in HSA solution, to describe it as 'inactive'.

Table 8. *Rate of oxidation of reduced glutathione in washed human erythrocytes, oxygenated in different protein solutions*

The protein solutions each contained 4 g. of the protein in 100 ml. of 0·75% NaCl. Washed, pooled cells were oxygenated at 37° in 1 vol. of the solution and GSH was then determined on the separated cells.

Solution	GSH oxidized/hr. (μmoles/100 ml. of cells) Average over 3 hr.			
	Expt. 1	Expt. 2	Expt. 3	Expt. 4
0·9% NaCl	16	23	35	25
Bovine-serum albumin	16	32	33	29
HSA	29	48	53	44
Bovine-serum γ-globulins	—	48	53	40

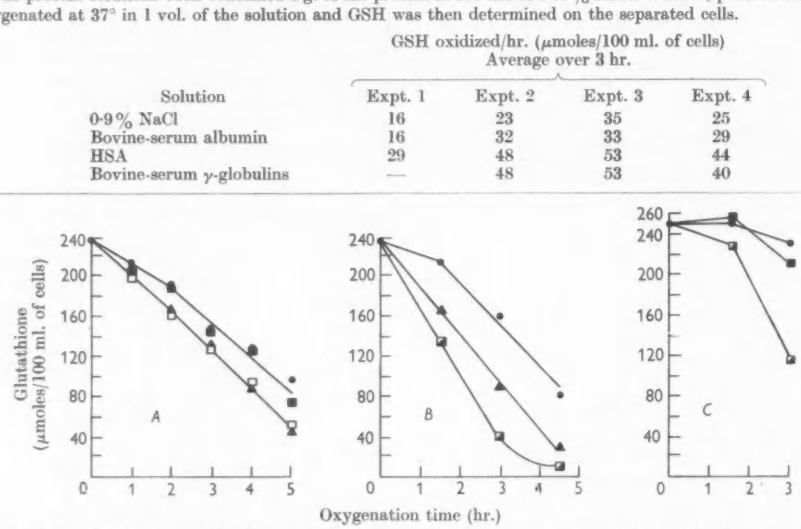

Fig. 3. Rate of oxidation of GSH in washed human erythrocytes oxygenated at 37° in the following media: ●, 0·9% NaCl; ▲, 0·75% NaCl containing HSA (4 g./100 ml.); ■, human dialysed serum containing NaCl (0·65 g./100 ml.), dialysis time, 24 hr.; □, the same dialysed serum previously heated at 70° for 1 min.; ◪, dialysed serum–NaCl, dialysis time, 44 hr. The erythrocytes used to obtain the data plotted in *A* and *B* were from the same batch of fresh, oxalated blood taken from a single donor. They were used 30 hr. after separating the plasma for *A* and 24 hr. later for *B*. The separated plasma from this same blood was used to prepare the dialysed serum used in *A*, *B* and *C*. The cells used for *C* were washed pooled erythrocytes and the dialysed serum (stored at 5°) was 24 hr. older than when used for *B*. The solution (1 vol.) was oxygenated at 37° with 1 vol. of the erythrocytes. At the stated times, samples of the mixtures were withdrawn and GSH determined in duplicate on the separated cells. Values given represent the mean of the two readings. For *C*, each oxygenation was also duplicated and the results are therefore the mean of four readings.

Table 9. *Relation between length of dialysis and heat treatment of human dialysed serum and its effect on the oxidation of reduced glutathione in washed human erythrocytes*

Washed pooled cells were oxygenated at 37° in 1 vol. of dialysed serum (prepared from fresh, pooled, oxalated plasma and containing 0·65 g. of NaCl/100 ml.) or the corresponding heated dialysed serum or 0·75% NaCl containing HSA (4 g./100 ml.). After oxygenation for the times shown, GSH was estimated on haemolysates of the separated cells. Each experiment relates to a different batch of dialysed serum and erythrocytes.

Expt. no.	Treatment of dialysed serum: Period of dialysis (hr.)	Treatment of dialysed serum: Time of heating at 70° (min.)	GSH (μmoles/100 ml. of cells) after oxygenation of cells with: Dialysed serum	GSH (μmoles/100 ml. of cells) after oxygenation of cells with: HSA solution	Time of oxygenation (hr.)
1	3	0	200		
		20	205	127	2
		30	175		
		40	137		
2	8	0	135		
		3	125	80	2
		10	95		
		15	70		
3	20	0	200	145	2·25
		3	152		
4	24	0	190	165	2
		0·5	160		

Dialysed serum (from pooled plasma; dialysis time, 20 hr.) remains active for at least 5 days after dialysis if stored at 5° and for 2–3 days if kept at 20°; plasma stored at −15° for 6 months and then dialysed yields an active dialysed serum.

In Fig. 4 the effect of diluting active dialysed serum (dialysis time, 5 hr.) with HSA solution, on the concentration of GSH in erythrocytes after various periods of oxygenation, is illustrated. Measured after the first 2 hr. of oxygenation, dialysed serum must be diluted more than four times before there is any significant fall in the GSH concentration from that found in the dialysed serum alone. After 3 or 4 hr. of oxygenation, when the difference between the GSH concentrations of the cells oxygenated in these two media has more than doubled, a fall in the concentration of GSH below that found in dialysed serum alone begins when the dialysed serum–HSA solution still contains 50–60 % of dialysed serum.

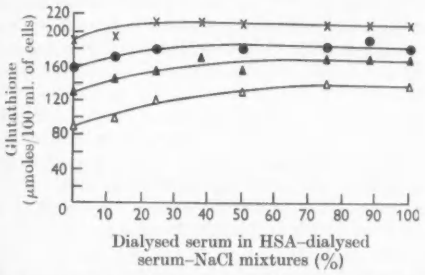

Fig. 4. Effect of diluting human dialysed serum with 0·75 % NaCl containing HSA (4 g./100 ml.) on the GSH concentration of washed pooled human erythrocytes, subsequently oxygenated in the mixture solution at 37° for 1 hr. (×), 2 hr. (●), 3 hr. (▲) and 4 hr. (△). Dialysed serum (dialysis time, 5 hr.) containing NaCl (0·65 g./100 ml.) was added to the HSA solution to give the required dilution of the dialysed serum: 1 vol. of each mixture was oxygenated with 1 vol. of washed erythrocytes and samples were withdrawn at the stated intervals for estimation of GSH. The mean of duplicate estimations is plotted. The dialysed serum was obtained from fresh plasma taken from a single donor.

The influence of temperature has been studied on the rate of oxidation of GSH in erythrocytes oxygenated in saline, active dialysed serum or HSA solution and the data from two experiments are illustrated in Fig. 5. The rates of oxidation of GSH at 37° are almost twice as great in the cells used in one of the experiments as the corresponding rates for the cells used in the other. On lowering the temperature from 37 to 23°, there is a substantial decrease in all the rates of oxidation. For the experiment with the lower rates at 37°, the rate for dialysed serum at 23° falls to 58 % of its former value and the rate for HSA solution to 42 %; in the other experiment, the rate for dialysed serum falls to 64 % and the rate for HSA

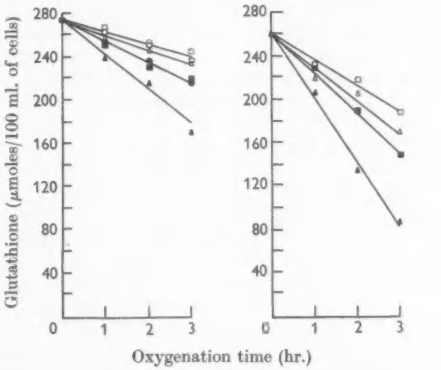

Fig. 5. Comparison of the rate of oxidation of GSH in washed pooled human erythrocytes oxygenated at 37° (●, ▲, ■) and 23° (○, △, □) in 0·9 % NaCl (●, ○), 0·75 % NaCl containing 4 g. of HSA/100 ml. (▲, △) and pooled human dialysed serum (dialysis time, 3 hr.) containing 0·65 g. of NaCl/100 ml. (■, □). The dialysed serum was obtained from fresh, pooled, oxalated plasma. The pooled washed erythrocytes were oxygenated in 1 vol. of the solution at the stated temperature, samples of the mixture were withdrawn every hour and GSH was estimated on haemolysates of the separated cells. Each graph represents a separate experiment with a different batch of erythrocytes.

Table 10. *Effect of length of dialysis and heat treatment of human dialysed serum on the comparative oxyhaemoglobin concentrations of oxygenated washed human erythrocytes*

Washed pooled erythrocytes were oxygenated at 37° in 1 vol. of the solution. Figures given were obtained after 1 hr. of oxygenation but there were no significant changes after 3 hr. The oxyhaemoglobin concentration of cells oxygenated in 0·9 % NaCl was taken as 100 %. The dialysed serum was obtained from fresh, pooled, oxalated plasma.

Treatment of pooled dialysed serum	Activity (see text)	Oxyhaemoglobin (%)
Dialysis for 8 hr.: unheated	Active	52
Dialysis for 8 hr.: heated for 1 min. at 70°	Active	22
Dialysis for 8 hr.: heated for 15 min. at 70°	Inactive	22
Dialysis for 30 hr.: unheated	Inactive	50

solution to 51 %. Thus in both experiments the proportional decreases resulting from lowering the temperature are of the same order and are greater for HSA solution than for dialysed serum.

The rate of oxidation of GSH in cells oxygenated in saline, which at 37° is equal to that in dialysed serum (see also Fig. 3*A*), also falls, on reducing the temperature to 23°, to the same extent as dialysed serum.

The oxyhaemoglobin concentration of cells after oxygenation in dialysed serum prepared in various ways has been measured (Table 10). Cells oxygenated with unheated dialysed serum whether active or inactive (in the sense defined above) show similar concentrations of oxyhaemoglobin and these are lower than those observed after oxygenation in HSA solution containing 4 g. of HSA/100 ml. (see Table 6). If the dialysed serum used has previously been heated, the oxyhaemoglobin concentrations found are much lower still and of the same order whether or not the dialysed serum remains active after the heat treatment used.

DISCUSSION

The wide variations found for the rates of oxidation of GSH in different batches of washed erythrocytes oxygenated in the same media and under the same conditions may be partly due to their different content of metals which catalyse the oxidation *in vitro*. One of these metals (see introduction) is copper, and cupric ions added to the medium do in fact increase the rate of oxidation in oxygenated cells. This effect is presumably exerted intracellularly because cupric ions are known to diffuse rapidly into human erythrocytes when added to blood either *in vivo* or *in vitro*. These ions gradually become bound in the cells because the amounts escaping when the cells are subsequently incubated with fresh plasma decrease the longer they are kept after exposure to the ions (Bush, Mahoney, Gubler, Cartwright & Wintrobe, 1956). One of the substances binding the added copper may be GSH since it reacts readily *in vitro* with cupric ions (Hopkins, 1929). If so, some of the endogenous copper found in erythrocytes (70–80 μg./100 ml. of cells) (Cartwright, 1950) may also be bound to GSH, the actual proportion of the total copper in the cells thus bound depending on the cell concentration of GSH. The higher the concentration of GSH the more catalyst for its own oxidation it would bind and this hypothesis would therefore explain the apparent paradox that the rate of oxidation of GSH tends to be greater the greater its concentration in the cells before oxygenation although the rate does not decrease during oxygenation as the concentration falls.

Variations in the rate of oxidation of GSH in different cells may also be due, in part, to variations in their content of substrates reducing triphosphopyridine nucleotide (Jocelyn, 1960) since these take part in the reverse reaction, the reduction of GSSG, and would therefore diminish the overall rate of oxidation of GSH. Such substrates (e.g. glucose 6-phosphate) would probably remain in the cells after washing (Aten & Hevesy, 1938).

In addition to a fall in the concentration of GSH in oxygenated erythrocytes, there is also a fall in the total glutathione (GSH + GSSG) content. The rate of both these falls is greater in the cells oxygenated in HSA solution than in the same cells oxygenated in saline. In both media, however, the rate of fall in total glutathione is much smaller than the rate of fall in GSH so that the cells oxygenated in HSA solution contain more GSSG than those oxygenated in saline for the same time. The fall in total glutathione in both media is therefore probably due to a fall in the GSSG level at a rate dependent on its rate of formation from GSH. This is further shown by the fact that when the formation of GSSG is diminished by adding glucose to the oxygenated cells (see introduction), the loss of total glutathione is found to be correspondingly smaller.

Of possible explanations for this fall in GSSG, the following appear to be excluded for the reasons given: (i) haemolysis: the degree of haemolysis of the cells after oxygenation is too small to account for the observed loss; in any case it is unaffected by alterations in the glucose content of the oxygenation medium; (ii) incomplete recovery: GSSG added to the haemolysates after oxygenation is quantitatively recovered as GSH; (iii) further oxidation, e.g. to the sulphonic acid: there is a continuing loss of total glutathione on incubating cells with a high GSSG content without further oxygenation; (iv) diffusion from the cells: the loss of total glutathione on incubating cells with a high GSSG content proceeds at the same rate whether the cells are incubated alone or suspended in saline.

The most likely explanation remaining is that the fall of GSSG is due to its participation in a slow non-oxidative reaction. Such a reaction may be with the SH group of proteins (Pr) within the erythrocyte to yield mixed glutathione–protein disulphide compounds:

$$\text{GSSG} + \text{Pr-SH} \rightarrow \text{GSS-Pr} + \text{GSH}$$

Evidence has been given for the occurrence of a similar reaction between protein SH groups and (as the non-protein disulphide) cystamine (Eldjarn & Pihl, 1956) or a derivative of diphenyl disulphide (Klotz, Ayers, Ho, Horowitz & Heiney, 1958). On the other hand, GSSG does not react *in vitro* with the SH group of HSA (Isles & Jocelyn, 1960).

When washed erythrocytes are treated with nitrogen in HSA solution, they show no fall in their GSH concentrations. Hence the additional fall of GSH in cells oxygenated in HSA solution over that of the same cells oxygenated in saline is due to a stimulant effect of HSA on the oxidation of GSH and not to any direct oxidation–reduction reaction between GSH and HSA.

Since it is unlikely that HSA can penetrate the erythrocyte membrane (Solomon, 1958), its stimulant effect on the oxidation of GSH is probably exerted extracellularly. A buffering action would appear to be ruled out since HSA has the same stimulant effect on the oxidation when added to Krebs–Ringer or Krebs–Ringer-phosphate solutions as when added to saline. Moreover, a buffering action would not explain the finding that bovine-serum albumin, which is chemically and antigenically similar to HSA (Boyd, 1954), does not give the effect. Bovine γ-globulins do so, however, and to the same extent as HSA so that certain structural features of the HSA molecule which are absent from bovine-serum albumin but present in bovine γ-globulins are needed for the effect to operate. The SH group cannot be one of these features since after treating HSA with iodine which oxidizes the SH group (Hughes & Straessle, 1950), the stimulant effect of the treated protein remains the same as before iodination; this contrasts with the finding (Saktor, O'Neill & Cochran, 1958) that the effect of HSA in stimulating oxidative phosphorylation in mosquito mitochondria is abolished by oxidation of the SH group with iodine.

If the rate of reaction between GSH and oxygen depended on the oxygen concentration within the cell, a plausible explanation for the stimulation by HSA could be given. This would envisage an effect on the cell membrane decreasing, below the value in saline, the gradient between the extracellular and the lower intracellular oxygen concentrations and thus increasing the latter.

The oxygen dissociation properties of oxyhaemoglobin in cells suspended in iso-osmotic saline are unlikely to be altered merely by adding HSA to the saline. Consequently, under these conditions, changes in the oxyhaemoglobin concentrations of the cells probably reflect changes in their oxygen concentrations. Cells oxygenated in HSA solutions have in fact lower oxyhaemoglobin concentrations than cells oxygenated in saline so that HSA may well have an effect on oxygen concentration gradient across the cell membrane. However, this effect would be a reduction in tension, and would not therefore account for its stimulation of the oxidation of GSH. In fact there would seem to be no relationship, not even an inverse one, between the oxyhaemoglobin concentration in the cell and the rate of oxidation of GSH. Thus HSA exerts its maximum effect on the oxidation at a concentration of 0·6–0·8 g./100 ml., whereas its effect in decreasing the oxyhaemoglobin concentration increases linearly up to at least 4 g./100 ml. Further, with differently treated batches of dialysed serum as the media, the same oxyhaemoglobin concentrations are associated in the same cells with widely different rates of oxidation of GSH.

The possibility that oxyhaemoglobin plays some essential but not rate-limiting role in the oxidation of GSH is not supported by the observation that the oxidation goes faster when the cells have been treated with carbon monoxide. The weight of this evidence however is complicated by the fact that carbon monoxide also raises the initial concentration of GSH as determined after subsequent precipitation of the proteins. Though this fact has not been explained, it is usually considered that the concentrations found in the treated cells represent the true endogenous concentrations (Numata, 1940; Thomson & Martin, 1959), the lower concentrations found in untreated cells being then ascribed to a loss, prevented by carbon monoxide, due to some kind of interaction with haemoglobin or oxyhaemoglobin during the precipitation of the proteins. On this explanation, there is no difference in the amount of GSH actually present in either the treated or untreated intact cells and the higher oxidation rate after treatment would suggest that haemoglobin or oxyhaemoglobin actually inhibits the oxidation in the untreated cells. But it is also possible that treatment with carbon monoxide does raise the concentration of GSH in the treated cells, e.g. by releasing it from some complexed form. In that case, the higher rates of oxidation observed would be due to the increased initial concentrations of GSH in the treated cells. These increased concentrations might result in a redistribution of copper and other heavy metals within the cell so that more became bound to GSH and hence available as catalysts to increase proportionately its rate of oxidation. In favour of this explanation is the fact that in the treated cells both the rates of oxidation in saline or HSA solution and the initial concentrations of GSH are all increased by about the same fraction (0·2–0·5) of their values in the untreated cells.

From the range found in normal serum (Stewart & Dunlop, 1958) dialysed serum contains 3·6–5·2 g. of HSA/100 ml., allowing for the 10% increase in volume which occurs on dialysis. When used after 3–24 hr. dialysis, as the medium for the oxygenation of washed erythrocytes, the rate of oxidation of GSH corresponds to the rate in saline not to the rate in HSA solution so that, present in the dialysed serum, there is some factor (or factors) inhibiting the effect of HSA.

Part of this factor is probably a protein because the HSA-inhibiting property (activity) is lost after heating this 3–24 hr.-dialysed serum at 70°. These conditions are unlikely to destroy a non-protein substance especially as the unheated dialysed serum retains activity for 2–3 days at room temperatures.

It also seems probable that a slowly diffusible cofactor is required since there is no activity when the dialysed serum is prepared after more lengthy dialysis (e.g. 44 hr.) of plasma. The connexion between length of dialysis and ease of heat inactivation at 70° could then be explained by the assumption that the factor required for activity is a complex between the protein and the cofactor which must be present in the dialysed serum in adequate concentration and which slowly dissociates on dialysis. Hence the shorter the dialysis the higher would be the concentration of this factor and consequently the longer the heat treatment needed to produce inactivation.

This explanation entails that after short dialysis the factor is present in dialysed serum in excess of the amount needed for full activity. This in fact appears to be the case because dialysed serum continues to inhibit the effect of HSA when diluted with HSA solution until its concentration in the mixture has fallen to 25–60 %. The actual point of dilution at which the inhibition begins to disappear probably depends on the magnitude of the stimulating effect of HSA itself. Consequently it may vary considerably both after different times of oxygenation of the same cells (as in Fig. 4) and also as between one batch of cells and another. There is also a further aspect to be considered. When dialysed serum, inactivated by long dialysis (and occasionally by heating), has been used as the medium for oxygenating cells, the rate of oxidation of GSH has usually been found to be much greater than the rate for the same cells in HSA solution (as in Fig. 3*B*). Hence in dialysed serum other proteins present may potentiate or supplement the effect of HSA on the cells so that the stimulative effect inhibited by the factor may considerably exceed that found for the same cells with HSA solution alone. Dilution of the dialysed serum with HSA will also dilute all the proteins other than HSA and this may reduce the stimulating effect as well as the concentration of inhibiting factor. The point of dilution with HSA where counteraction of the stimulating effect begins to disappear cannot therefore be used to measure factor activity.

Whether lowering the temperature affects the inhibiting activity is also uncertain. The rates of oxidation of GSH in cells oxygenated in saline and dialysed serum both fall by the same amount and therefore there is no evidence that the inhibiting is preferentially affected by temperature.

The existence in dialysed serum (and therefore serum) of a factor inhibiting the effect of HSA on the oxidation of GSH in oxygenated erythrocytes may be of some relevance in physiology. A requirement for oxyhaemoglobin has not been demonstrated so that it is possible that both the effect and also its counteraction by a serum factor would also be observed in cells containing GSH from other tissues.

In the presence of glucose, however, net oxidation of GSH cannot normally be shown, because the reduction of the GSSG formed by reduced triphosphopyridine nucleotide (TPNH) and glutathione reductase is rapid enough to maintain the glutathione of the tissues predominantly as GSH (Bhattacharya, Robson & Stewart, 1955). TPNH also predominates over its oxidized form (TPN) (Glock & McLean, 1955; Bassham, Birt, Hems & Loening, 1959) and it is likely that the rate at which it can be oxidized limits glucose metabolism by the TPN-specific pentose phosphate pathway (de Duve & Hers, 1957). Glutathione reductase is an abundant enzyme with high activity in most tissues (Rall & Lehninger, 1952) and, like the pentose phosphate pathway enzymes, is present in cells mainly in the extraparticulate fraction (Kinoshita & Masurat, 1957) so that it is likely and in some tissues (e.g. the bovine lens) probable, (Kinoshita & Masurat, 1957) that the reduction of GSSG provides an important route for the oxidation of TPNH (see also Waley, 1959*a*). The rate of this reaction would be limited by the availability of GSSG, i.e. by the rate of oxidation of GSH (the 'missing step' of Waley, 1959*b*).

The range for the rate of oxidation of GSH in washed erythrocytes oxygenated in saline (and therefore active dialysed serum) is 5–50 μmoles of GSH/hr./100 ml. of cells (Table 1). Reduction of GSSG by reduced diphosphopyridine nucleotide is negligible (Racker, 1955*a*; Francoeur & Denstedt, 1954) so that probably in the presence of glucose all the GSSG formed is converted back into GSH by TPNH. If the oxidation of TPNH is the rate-limiting reaction this would enable 1·2–12·4 μmoles of glucose (0·2–2·1 mg.)/100 ml. of cells to proceed through the pentose phosphate pathway per hour taking this pathway to involve the reduction of two molecules of TPN/molecule of glucose. The mean consumption rate of glucose in normal erythrocytes aerated in plasma at 37° is 31–43 mg./100 ml. of cells/hr. (Buffa, Maraini & Chiarle, 1959) giving a range of 0·5–6·7 % of glucose metabolized to pentose phosphate. This estimate agrees with the conclusion of Coxon & Robinson (1956) that the pathway is not important quantitatively in erythrocytes. However, if the rate of oxidation of GSH in erythrocytes could be stimulated by HSA *in vivo*, then, from the data for the initial rates *in vitro* (e.g.

Fig. 1), this proportion of glucose metabolized to pentose phosphate would have to be increased at least two- to three-fold.

In other tissues (e.g. liver and spleen), much higher values have been given for glucose metabolized by this pathway (see Siperstein, 1959; Dickens, 1958). In these tissues the concentration of GSH is also higher than in erythrocytes (see Jocelyn, 1959) and, for rat and rabbit liver, its rate of oxidation in saline (and hence possible stimulation by HSA) is over 10 times as fast (Hopkins & Elliott, 1932). Accordingly, there would seem to be the prospect that human-serum albumin and a serum factor, acting antagonistically on the rate of oxidation of glutathione, might form part of a general mechanism for regulating extracellularly the tissue utilization of glucose along the pentose phosphate pathway.

SUMMARY

1. When intact washed human erythrocytes are oxygenated, e.g. in saline (0·9% sodium chloride solution), glutathione is converted into its oxidized form. Some of this is then slowly lost by an unknown route.

2. The rate of oxidation of glutathione in oxygenated erythrocytes is greater when the oxygenation is carried out in human-serum-albumin or bovine-γ-globulin solutions than when it is carried out in bovine-serum-albumin or saline solutions.

3. In human-serum-albumin solutions, the maximum stimulant effect is obtained at a protein concentration of 0·6–0·8 g./100 ml. and this is unaffected by previous oxidation of the albumin thiol group.

4. The rate of oxidation of glutathione is unrelated to the oxyhaemoglobin level of the cells during the oxygenation.

5. The rate of oxidation of glutathione in erythrocytes oxygenated either in saline or in human-serum-albumin solution is increased as a result of treating the cells with carbon monoxide.

6. The rate of oxidation of glutathione in erythrocytes oxygenated in dialysed serum, prepared from human plasma after dialysis for 3–24 hr., is similar to the rate for the same cells oxygenated in saline.

7. If this dialysed serum is preheated at 70° or prepared after dialysis of plasma for e.g. 44 hr., the rate is then similar to that found for the same cells oxygenated in human-serum-albumin solution. The time of heating required at 70°, to change the rate of oxidation in the same cells from that in saline to that in human-serum-albumin solutions, increases as the time of dialysis decreased.

8. The rate of oxidation of glutathione in oxygenated erythrocytes is reduced by lowering the temperature by 14°, more so when the oxygenation is carried out in human-serum-albumin solution than when it is carried out in saline or dialysed serum.

9. The effect of progressively diluting dialysed serum with human-serum-albumin solution on the rate of oxidation of glutathione in erythrocytes oxygenated in the dialysed serum–albumin mixtures has been studied.

10. The evidence given suggests that human serum contains a heat-labile protein and a slowly diffusible substance which together inhibit the stimulant effect of human-serum albumin on the oxidation of glutathione in erythrocytes.

11. The mechanism of this inhibition and its possible role in metabolism are discussed.

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The Sulphatase of Ox Liver

7. THE INTRACELLULAR DISTRIBUTION OF SULPHATASES A AND B*

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Previous papers in this series (Roy, 1953*a*, *b*, 1954*a*, 1956) have shown that ox liver contains three arylsulphatases, sulphatases A, B and C, which differ both in their general properties and in their intracellular localizations. Sulphatase C apparently occurs only in the microsomes (Roy, 1956, 1958), whereas sulphatases A and B appear in several cell fractions. It was originally suggested that they occurred predominantly in the mitochondria and microsomes of rat liver (Roy, 1954*b*), but Viala & Gianetto (1955) showed that their distribution was consistent with their arising from the lysosomes, a conclusion that was supported by subsequent work in other species (Roy, 1958). However, these investigations all suffered from the disadvantage that sulphatases A and B were determined as one unit, the two individual enzymes not being separately determined. It is obvious that such studies could give misleading results and that important differences in the localizations of the two enzymes might not be detected. Unfortunately, the separate determination of sulphatases A and B in a mixture is a matter of some difficulty, especially when the situation is further complicated by the simultaneous presence of sulphatase C.

* Part 6: Roy (1957).

This paper describes the results of an investigation into the intracellular distribution of sulphatases A and B in ox liver. This species was chosen for study as in it the differences between these two enzymes are greater than those between the corresponding enzymes of the rat (Dodgson, Spencer & Thomas, 1955) and, further, they can readily be separated by their different solubilities in water, sulphatase A being insoluble (Roy, 1953*b*), whereas sulphatase B is soluble (Roy, 1954*a*).

A preliminary account of this work was presented at the Canberra meeting of the Australian Biochemical Society in January 1960.

EXPERIMENTAL

Cell-fractionation and general techniques. The samples of ox liver were obtained at the slaughter house within a few minutes of the death of the animal and were immediately placed in 0·25 M-sucrose at 0° for transport to the laboratory. Further treatment was commenced within 30 min. of death.

The mitochondria and microsomes were isolated from a 10% (w/v) dispersion of ox liver in 0·25 M-sucrose by the standard technique of Hogeboom (1955), as described by Roy (1958) except that the mitochondria were washed only once. The particles were extracted by suspending them in the appropriate solvent at 0° (1 ml./g. of liver) and then

centrifuging for 1 hr. at 50 000 *g*. Dialyses were carried out in Visking cellophan tubing against repeated changes of 100 vol. of water at 0° for the times specified below.

Enzyme assays. The general technique was a simplification of that originally described (Roy, 1953*a*) with dipotassium 2-hydroxy-5-nitrophenyl sulphate (nitrocatechol sulphate, NCS) as the substrate. The reaction mixture had a volume of 0·8 ml. and contained 0·2 ml. of enzyme preparation. The exact composition of the various mixtures is described below. After incubation for 1 hr. at 37° proteins were precipitated by the addition of 3 ml. of 0·1 M-trichloroacetic acid and removed by centrifuging. A portion (3 ml.) of the supernatant solution was pipetted into 5 ml. of 2·5 N-NaOH and the extinction of the resulting solution was determined in the Unicam SP. 600 spectrophotometer at 515 mμ. The amounts of 4-nitrocatechol present were determined from suitable calibration curves.

Trichloroacetic acid was used as protein precipitant instead of the phosphotungstic acid of the original method in order to avoid the formation of the troublesome precipitate which appeared with the latter in high concentrations of electrolytes (Roy, 1954*a*). Similarly, 2·5 *N*-NaOH was used in place of alkaline quinol (Roy, 1953*a*) as the colour of the 4-nitrocatecholate ion was stable at the protein concentrations used in the present series of determinations.

The general conditions of the assays were as follows. In preliminary studies of the cell fractionation and the subsequent treatment of the fractions: 15 mM-NCS in 0·125 M-acetic acid–sodium acetate buffer (acetate buffer), pH 6·0; for the assay of sulphatase A: 3 mM-NCS in 0·125 M-acetate buffer, pH 5·0 (Roy, 1953*b*); for the assay of sulphatase B: 0·06 M-NCS in 0·125 M-acetate buffer, pH 5·9 (Roy, 1954*a*, 1955), or 15 mM-NCS in 0·5 M-acetate buffer, pH 5·6. Any adjustments of pH which were required were made with 2 N-acetic acid or 2 N-NaOH.

A few determinations were also carried out with potassium *p*-nitrophenyl sulphate (NPS) as substrate. The general procedure was similar to that described above except that 3 ml. of the deproteinized reaction mixture was pipetted into 5 ml. of M-Na_2CO_3 for colour development and the extinction of the resulting solution was determined at 400 mμ. The detailed conditions used in these assays are described in the following section.

Protein determinations. These were carried out by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine-serum albumin as standard.

RESULTS

Preliminary separation of sulphatases A and B. The mitochondria or microsomes were suspended in water to give a concentration corresponding approximately to 1 g. of liver/ml. and kept at 0° for from 4 to 18 hr. Such variations in time were without effect on the subsequent behaviour of the enzymes. The response of the arylsulphatase activity in such suspensions to changes in substrate concentration is shown in Fig. 1. The suspensions were then centrifuged for 1 hr. at 50 000 *g*: the supernatants were decanted and retained (the aqueous extracts) and the residues were resuspended in water and made up to the original volume (the insoluble residues). The influence of changes in substrate concentration on the arylsulphatase activities of these fractions is also shown in Fig. 1. It is obvious that the aqueous extracts and the insoluble residues, both from the mitochondria and the microsomes, differ

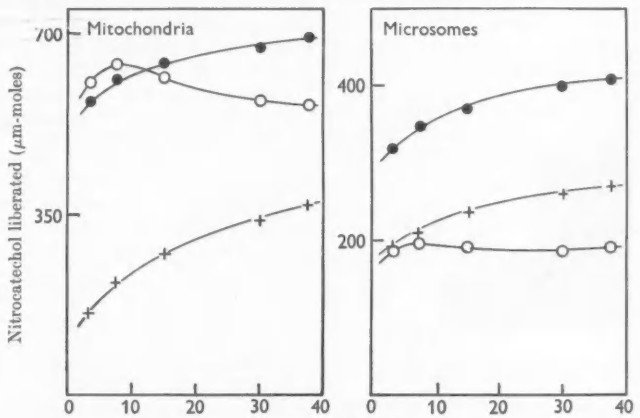

Fig. 1. Effect of changes in substrate concentration on the arylsulphatase activity of ox-liver mitochondria, microsomes and fractions thereof. Volume of reaction mixture, 0·8 ml., containing enzyme corresponding to approx. 150 mg. of liver; pH 6·0 in 0·125 M-acetate buffer. Incubated for 1 hr. at 37°. ●, Mitochondria or microsomes; ○, aqueous extracts; +, insoluble residues from the mitochondria or microsomes prepared as described in the text.

considerably in their properties and that whereas the former behave as preparations of sulphatase A containing small amounts of sulphatase B the latter behave as typical preparations of sulphatase B.

The efficiency of the extraction of the sulphatase activity was examined only with the mitochondria, as not only did the presence of sulphatase C in the microsomes complicate the situation therein but these particles did not sediment readily after extraction with water so that it was impossible to separate quantitatively the soluble material from the sediment. The results of a typical experiment are shown in Fig. 2. The major part of the soluble-sulphatase activity is removed by one extraction with water and the remainder almost all appears in the second extract as the third contains only traces of sulphatase activity.

Sulphatases of the aqueous extract. When the aqueous extracts from either the mitochondria or the microsomes were dialysed for 2–3 days a copious flocculent precipitate appeared. This contained about 90 % of the sulphatase activity of the extract. The remaining 10 % of the sulphatase was not precipitated even on prolonged dialysis. The precipitate was centrifuged off, washed with water and suspended in half the original volume of 0·5 M-sodium acetate, pH 6. After standing overnight the remaining insoluble material was centrifuged off and the supernatant dialysed for 4 hr. to decrease the concentration of acetate. The resulting solution contained between 60 and 80 % of the original soluble-sulphatase activity: most of the remainder was present in the residue from the sodium acetate extraction, and although it could only be extracted therefrom with difficulty its properties agreed in every other respect with those of the enzyme soluble in sodium acetate.

The effect of changes in substrate concentration on the arylsulphatase activities of such soluble-enzyme preparations from the mitochondria and the microsomes is shown in Fig. 3. It is obvious that the preparations from these two sources are identical with one another and with a preparation of sulphatase A obtained by the methods described by Roy (1953*b*). In all other respects studied such enzyme preparations from mitochondria or microsomes behaved as typical preparations of sulphatase A: the values of K_m, the shapes of the pH–activity curves and the anomalous effects of changes in enzyme concentration were all indistinguishable from those of purified preparations of sulphatase A (Roy, 1953*b*). Determinations of the specific activities of the preparations showed them to be of a purity comparable with that of sulphatase A-1 (Roy, 1953*b*).

The small amounts of arylsulphatase which were not precipitated by dialysis against water were quite different from the above-described prepara-

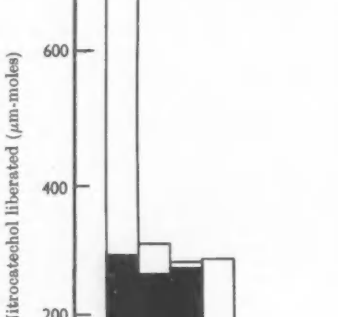

Fig. 2. Extraction of arylsulphatases from ox-liver mitochondria by successive treatments with water (1–3) and 0·15 M-KCl (4–6). General conditions of assay were as given in Fig. 1 except that the substrate concentration was fixed at 15 mM-NCS. Open areas represent the arylsulphatase activity which had been extracted by the solvent in question; filled areas indicate the arylsulphatase activity remaining in the residue.

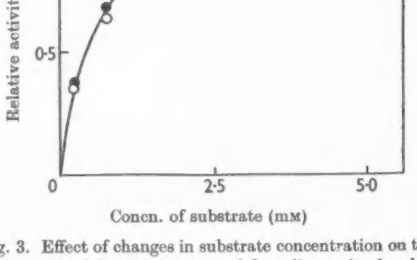

Fig. 3. Effect of changes in substrate concentration on the activity of sulphatase A prepared from liver mitochondria (●) or microsomes (○). Conditions were as given in Fig. 1 except that the pH was 5·0 in 0·125 M-acetate buffer. The line is the appropriate curve for an authentic preparation of sulphatase A (replotted from Roy, 1953*b*).

tions and corresponded to a sulphatase B, indistinguishable from the enzymes described below. The presence of a sulphatase B in the aqueous extracts was confirmed by paper electrophoresis as previously described (Roy, 1954*a*).

Sulphatase of the insoluble residue. The data shown in Fig. 1 indicated that the insoluble residues of both the mitochondria and the microsomes must contain a sulphatase B, presumably along with a sulphatase C in the latter. Soluble preparations of sulphatase B were obtained from the insoluble residues by a number of techniques.

When the suspension, buffered by the addition of 0·1 vol. of 0·2M-sodium citrate buffer, pH 6·2, was stirred vigorously at room temperature for 30 min. with 1 vol. of butan-1-ol and the resulting emulsion was broken by centrifuging, a considerable proportion of the sulphatase activity was found in the aqueous phase. This solution, the butanol-solubilized enzyme, contained (after dialysis to remove any butanol) about 50 and 25% of the sulphatase activity of the insoluble residues from the mitochondria and the microsomes respectively.

When the suspensions of the insoluble residues were taken to pH 4·2–4·5 with 2N-acetic acid, incubated at 37° for 15 min. and then centrifuged, the solutions so obtained, the acetate-soluble enzymes, contained sulphatase activity in amounts similar to those in the corresponding butanol-solubilized enzymes.

Finally, when the insoluble residues were suspended in 0·15M-KCl in place of water and then centrifuged, the resulting extract, the KCl-soluble enzyme, contained considerable amounts of sulphatase activity, about 70 and 50% from the mitochondrial and microsomal residues respectively. The efficiency of the extraction by KCl is shown in Fig. 2: again it is obvious that the major part of the sulphatase is removed in the first and second extractions, the third containing only traces of activity. Further small amounts of sulphatase could be obtained from the residue remaining after the KCl extraction by treatment with butanol as described above but it was not possible to obtain all the sulphatase activity from the insoluble residues in a soluble form.

In Fig. 4 is shown the effect of changes in substrate concentration on the arylsulphatase activity of these three types of preparation (butanol-solubilized, acetate-soluble and KCl-soluble) and on the sulphatase B activity of the aqueous extracts of the mitochondria and microsomes and of the residue from the KCl extraction of the mitochondria. These assays were carried out in 0·5M-acetate buffer, not in 0·125M-acetate buffer as previously (Roy, 1954*a*) because it is now known that the behaviour of impure preparations of sulphatase B is greatly influenced by the concentration of the acetate buffer used (Dodgson & Wynn, 1958): in particular many of the irregularities in the behaviour of relatively impure preparations disappear in more concentrated buffers. It is obvious from the results shown in Fig. 4 that these preparations of sulphatase B are identical with one another and with a purified preparation of sulphatase B. The pH–activity curves, determined in 0·5M-acetate buffers at a substrate concentration of 15 mM-NCS, were similarly indistinguishable from one another. The specific activities of the various

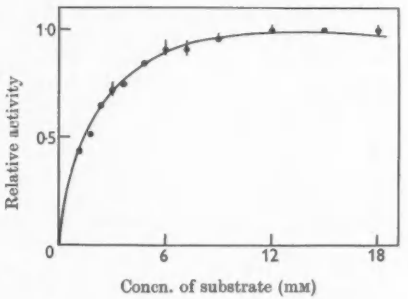

Fig. 4. Effect of changes in substrate concentration on the sulphatase B activity of various preparations obtained as described in the text. The points on the graph indicate the mean values and the vertical lines show the ranges of the activities where these are greater than the diameter of the points. The line is the appropriate curve for an authentic preparation of sulphatase B-2 (Roy, 1954*a*). General conditions of assay were as given in Fig. 1 except that the pH was 5·6 in 0·5M-acetate buffer.

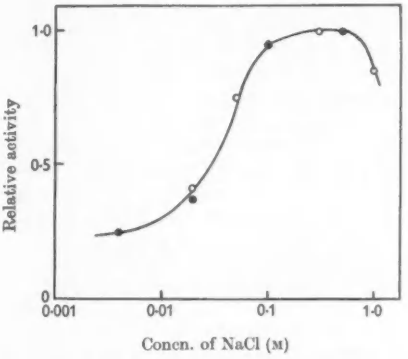

Fig. 5. Effect of varying concentrations of NaCl on the hydrolysis of NPS by sulphatase B. Volume of reaction mixture, 0·8 ml.; substrate concentration 30 mM-NPS in 0·5M-acetate buffer, pH 5·9. Incubated for 1 hr. at 37°. ●, Butanol-solubilized enzyme; ○, data of Webb & Morrow (1959) replotted to allow comparison.

preparations showed them all to be rather purer than sulphatase B-2 (Roy, 1954*a*).

Webb & Morrow (1959, 1960) have recently shown that, contrary to previous views, sulphatase B will hydrolyse NPS when the reaction mixture contains Cl^- ions. This is also true for preparations of the butanol-solubilized enzyme. Fig. 5 shows the dependence of the hydrolysis of NPS upon the presence of Cl^- ions and includes comparable data replotted from Webb & Morrow (1959). It is obvious that the responses of the two preparations are identical.

Quantitative distribution of sulphatases A and B. The mitochondrial and microsomal fractions were prepared as usual and each was successively extracted twice with water and twice with 0·15 M-KCl. These extracts were dialysed for 3 days, the resulting precipitates were removed by centrifuging and then were resuspended in water. The suspensions so obtained and the supernatants from the centrifugings were assayed for sulphatases A and B respectively. The residues remaining after the extractions of the mitochondria and microsomes were suspended in water, dialysed and assayed for sulphatase B.

The results of a typical experiment are shown in Table 1. This again shows that most of the sulphatase A is removed by the first treatment with water, both from the mitochondria and the microsomes, although the extraction is apparently less complete from the latter, probably because they pack only extremely loosely on centrifuging. About 20% of the sulphatase B was also found in the first aqueous extract but virtually none in the second: this would suggest that this fraction of the sulphatase B originated from cell particles that had been damaged during their isolation. The major part of the remainder of the sulphatase B appeared in the first KCl extract.

With the microsomes a considerable proportion of the sulphatase activity remained in the insoluble residue. Some of this must be due not to sulphatase B but to the sulphatase C which is localized in the microsomes. It is difficult to assess accurately the contribution due to the latter but from sulphatase determinations made in the presence of 0·01 M-KH_2PO_4, which is a powerful inhibitor of sulphatase B (Roy, 1954*a*) but not of sulphatase C (Roy, 1956), it seems probable that from 60 to 70% of the total insoluble sulphatase activity in microsomal residue is due to sulphatase C.

An exact value for the ratio of the activity of sulphatase A to sulphatase B in the two cell fractions is difficult to obtain because of this doubt about the true value of the sulphatase B activity in the microsomal residue. If, however, it be taken that 65% of the apparent sulphatase B activity is in fact due to sulphatase C then the ratio of A to B in the mitochondria is 0·30, whereas that in the microsomes is 0·26. The ratios are therefore very similar, if not identical, in the two fractions. These values for this ratio should also be compared with that of 0·26 obtained for the activities of the two enzymes purified to stages A-1 (Roy, 1953*b*) and B-1 (Roy, 1954*a*) respectively from a sample of acetone-dried liver prepared from the same animal as used in the above-described cell-fractionation studies. The use of the same animal for both types of experiment was essential as there are considerable variations of the ratio of sulphatase A to sulphatase B in different animals.

DISCUSSION

First, it must be pointed out that although the cytological significance of the mitochondrial and microsomal fractions isolated from ox liver was not investigated and the two fractions were defined purely on the basis of their sedimentation rates there is no reason to believe that they are of a different nature from the corresponding fractions of rat liver.

The results described above conclusively show that enzymes having the properties of sulphatases A and B can be obtained by identical procedures from both the mitochondrial and microsomal fractions prepared by standard procedures from a dispersion of ox liver in 0·25 M-sucrose. Further,

Table 1. *Extraction of sulphatases A and B from ox-liver mitochondria*

Details of the procedure are given in the text. Sulphatase A activity is expressed as μg.$^{\frac{1}{2}}$ of nitrocatechol liberated/hr. from 3 mM-NCS at pH 5·0 in 0·125 M-acetate buffer (Roy, 1953*b*), and sulphatase B activity as μg. of nitrocatechol liberated/hr. from 15 mM-NCS in 0·5 M-acetate buffer, pH 5·6.

	Mitochondria		Microsomes	
	A	B	A	B
First aqueous extract	3980	2940	1140	1610
Second aqueous extract	135	120	427	0
First KCl extract	87	8250	57	2950
Second KCl extract	33	625	64	487
Residue from the extractions	—	2390	—	4500*

* This figure is falsely high because of the presence of sulphatase C in the microsomes.

the ratios of sulphatase A to sulphatase B are similar in the mitochondria and the microsomes. These observations confirm the view that sulphatases A and B occur in the lysosomes, or at least in some particle which distributes itself between the mitochondrial and microsomal fractions as these are normally obtained. An alternative explanation would be that the two enzymes were in fact present in the mitochondria and microsomes themselves but, although small amounts could occur therein and escape detection by the methods used above, it would seem that this possibility can be discounted as it is unlikely that the two enzymes would occur in the same proportions in the two cell fractions and that they could separately be prepared therefrom by identical and mild techniques: the very different structures of the mitochondria and microsomes would make this latter suggestion extremely unlikely. It seems justifiable to conclude therefore that sulphatases A and B are present in some cell fraction which contaminates the mitochondrial and microsomal fractions, as do the lysosomes. Although other similar particles may exist, the strong similarities between the behaviour of the sulphatases and the behaviour of the typical lysosomal enzyme acid phosphatase (Viala & Gianetto, 1955) make it very likely that the particles involved are indeed lysosomes.

There are, however, important differences in the ease of extraction of sulphatases A and B. Sulphatase A can readily be extracted from the particles by water, in contrast with sulphatase B, which requires treatment with iso-osmotic potassium chloride, with butan-1-ol or with a lowered pH to effect solution. This difference could have several possible explanations. First, both sulphatases A and B might initially be released in a soluble form by treatment with water and only the latter might subsequently be adsorbed by particulate material. Such a situation seems unlikely as it is hardly to be expected that sulphatase B would be virtually quantitatively adsorbed by both the mitochondria and the microsomes, having regard to their very different structures. One of two other possibilities seems more probable: either the two enzymes are localized differently within the lysosomes or the lysosomes are heterogeneous, some containing sulphatase A, some sulphatase B. If the first possibility be correct then it is likely that sulphatases A and B respectively occur in the sap and insoluble framework (de Duve, Pressman, Gianetto, Wattiaux & Appelmans, 1955) of the lysosomes; if the second be correct then sulphatase A must probably occur in the sap of the lysosomes, as originally defined, and sulphatase B occur in some related but structurally different particle. It is impossible to distinguish between these possibilities at present, but evidence pointing to a heterogeneity in the lysosomes is accumulating. Beaufay, Bendall, Baudhuin, Wattiaux & de Duve (1959) have suggested that there may be as many different types of lysosome as there are lysosomal enzymes and, further, that uricase may be associated either with another type of particle or with the insoluble framework of the lysosome. Again, Conchie & Levvy (1960) have concluded that the results of their investigation of the distribution of glycosidases in mammalian liver can best be explained on the basis of the occurrence of a number of different types of lysosome-like particle. It is obvious that the final solution to these problems will be possible only with the development of even more refined techniques of fractional centrifuging than those used by Beaufay *et al.* (1959).

It should be noted that the availability of methods for the preparation of ox-liver mitochondria on a large scale (Beinert, 1957) makes the above-described techniques suitable for the preparation of sulphatases A and B. The two enzymes are readily obtained in a fair degree of purity and the tedious and expensive preparation of large amounts of acetone-dried liver is avoided. Further, the fact that the relative amounts of sulphatases A and B obtained by the two very different types of procedure are similar gives added weight to the view (Roy, 1954*a*) that both enzymes are true components of liver tissue and not artifacts.

SUMMARY

1. It has been shown that sulphatases A and B occur in the same proportions in both the mitochondrial and microsomal fractions of ox liver.

2. Sulphatase A can be obtained from both fractions by extraction with water. Such extracts also contain traces of sulphatase B, which may originate from cell particles damaged during the isolation procedure.

3. Sulphatase B cannot be extracted from the particles by water but can be obtained in solution by extraction with 0·15M-potassium chloride, by treatment with butan-1-ol or exposure to a lowered pH.

4. The properties of sulphatases A and B prepared as described above are identical with those of the corresponding enzymes obtained by fractional precipitation with acetone and ammonium sulphate.

5. It is concluded that both sulphatases A and B occur in the lysosomes but are either differently localized within these particles, sulphatase A occurring in the sap and sulphatase B in the insoluble framework, or the lysosomes themselves are heterogeneous, some containing sulphatase A, some sulphatase B.

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The Influence of the Induction of Alloxan-Diabetes on the Incorporation of Amino Acids into Protein of Rat Diaphragm

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Insulin *in vitro* stimulates the incorporation of labelled amino acids into protein of isolated diaphragm from the normal rat (Sinex, MacMullen & Hastings, 1952; Krahl, 1953; Manchester & Young, 1958; Wool & Krahl, 1959) and from the hypophysectomized or adrenalectomized rat (Kostyo, 1959; Manchester, Randle & Young, 1959). On the basis of these and other observations, it is to be expected that the rate of incorporation of labelled amino acids into protein of isolated diaphragm from the alloxan-diabetic rat would be less than that of diaphragm from the normal rat, and that treatment of the alloxan-diabetic rat with insulin would restore the rate of incorporation to normal. This possibility, in both the newly diabetic and the 'long-term' diabetic animal, has now been investigated. The ability of plasma from rats, in various stages of alloxan-diabetes, to stimulate the uptake of glucose and incorporation of amino acid into protein by diaphragm from normal rats has also been studied.

MATERIALS AND METHODS

Animals. Albino Wistar female rats of about 130 g. wt. were used throughout. They were fed *ad libitum* a standard laboratory diet (Bruce & Parkes, 1949) and had free access to water at all times.

Alloxan-diabetes. Diaphragm was taken from rats at two widely different periods after they had been treated with alloxan. What are termed 'short-term' alloxan-diabetic rats had received alloxan 2 days (48 hr.) before removal of the diaphragm; 'long-term' alloxan-diabetic rats had received alloxan at least 2 weeks previously. In both instances the rats were starved 24 hr. before a subcutaneous injection of alloxan was given [alloxan monohydrate, British Drug Houses Ltd., 200 mg./kg. in 1% (w/v) NaCl]. Under these conditions an intense hyperglycaemia developed, and 48 hr. after the administration of alloxan the blood sugar usually exceeded 500 mg./100 ml. even though food had been withdrawn 24 hr. previously.

No deaths usually occurred within 48 hr. of the administration of alloxan, but unless the rats were given insulin daily for at least a week after the injection of alloxan, many deaths took place within 2–7 days of this treatment. The following procedure was therefore adopted for the preparation of 'long-term' diabetic animals: each animal received 2 units of protamine–zinc (PZ) insulin (Burroughs Wellcome and Co. Ltd.) subcutaneously on the evening of days 2–7 after treatment with alloxan. On days 8–10 the daily dose of insulin was decreased to 1 unit and on days 11–12 to 0·5 unit. During the period of days 2–10 inclusive, drinking water was replaced by 1% (w/v) glucose solution in order to prevent hypoglycaemia. After day 12, treatment with insulin was discontinued unless the animal showed signs of impending diabetic coma, when soluble insulin (1 unit/day) was administered for a day or two. This procedure made possible the survival, 14 days after the administration of alloxan, of about 60% of the number treated. All these animals when deprived of insulin for 2 or more days had non-fasting blood-sugar levels above 500 mg./100 ml., but after the withdrawal of food the blood sugar dropped to about 215 mg./100 ml. (range 100–500 mg./100 ml.). At

this time they possessed very little subcutaneous and retroperitoneal fat.

Hypophysectomy. Rats were hypophysectomized by the para-pharyngeal route under ether anaesthesia. Success of the operation was judged, 10 or more days later, by failure of the animals to gain weight. Hypophysectomized animals, which had been treated with alloxan (200 mg./kg. subcutaneously), as well as control hypophysectomized animals, were given 1% glucose solution to drink instead of water.

Adrenalectomy. Bilateral adrenalectomy was carried out under ether anaesthesia through a mid-line dorsal incision. The adrenalectomized rats were given 1% NaCl to drink. Alloxan (200 mg./kg.) was injected subcutaneously 48 hr. after adrenalectomy. As with the hypophysectomized rats, the alloxan-treated adrenalectomized rats and the control adrenalectomized animals were given 1% glucose solution to drink.

Incubation of diaphragm and preparation of protein samples. The methods for the removal and incubation of diaphragm, and the preparation and assessment of the content of radioactivity of samples of protein, were those described by Manchester & Young (1958). Diaphragm (2 hemidiaphragms in 2 ml.) was incubated for 2 hr. at 37° in the bicarbonate-buffered medium of Gey & Gey (1936), gassed with $O_2 + CO_2$ (95:5), usually in the presence of 1 μmole/ml. of [1-^{14}C]glycine (specific activity, about 400 μc/m-mole). A radioactive protein hydrolysate, prepared by the acid hydrolysis of protein containing a large number of ^{14}C-labelled amino acids which had been obtained from *Chlorella* grown in the presence of $^{14}CO_2$, was used at a concentration of about 0·01 mg./ml. and a specific activity of about 37 μc/mg. Radioactive materials were obtained from The Radiochemical Centre, Amersham, Bucks. In some instances glucose was added to the medium to give a final concentration of 2·5 mg./ml.

Adrenaline. Adrenaline (British Drug Houses Ltd.) was dissolved in a small quantity of 0·1 N-HCl and diluted with Gey buffer to a concentration of 1 μg./ml.

Measurement of glucose uptake by diaphragm. Uptake of glucose by diaphragm was measured as the amount of glucose which disappeared from the medium. Glucose was determined by the colorimetric method of Somogyi (1945).

Blood plasma. Blood was collected from rats, after decapitation, in heparinized beakers and centrifuged. For the estimation of insulin activity pooled blood plasma, usually from six rats, was diluted with Gey buffer to 25%, and glucose added to the diluted plasma in such amount as to make the final glucose concentration 2·5 mg./ml. The method was essentially that of Randle (1956).

It has been reported (Bornstein & Park, 1953; Whitney & Young, 1957) that blood plasma from alloxan-diabetic rats, in contrast to that of normal rats, does not stimulate the uptake of glucose by isolated diaphragm from normal rats. But if the plasma is frozen and thawed three times insulin activity may appear (Randle, 1955; Hendley, Bregman & Krahl, 1957). Plasma of alloxan-diabetic rats may therefore contain insulin which is rendered biologically inactive by the presence of an antagonist. Freezing and thawing, by making the postulated inhibitor ineffective, enables the insulin present in the plasma to be detected by its effect on the uptake of glucose by diaphragm from the normal rat. Frozen and thawed plasma was prepared by freezing the fresh plasma in a mixture of acetone and solid CO_2, and thawing at 37°, three times altogether.

Acid-ethanol extracts of precipitated plasma proteins contain material with insulin-like activity which is believed to be at least a significant proportion of the total insulin present in the plasma (Taylor, 1959). We have used the method of Taylor (1959) to determine the amount of extractable insulin in plasma from alloxan-diabetic rats. To a sample of plasma (5 ml.) an equal volume of 10% (w/v) trichloroacetic acid was added. The precipitate was collected by centrifuging and washed with 5 ml. of 10% trichloroacetic acid. It was then extracted twice with about 5 ml. of acid–ethanol [conc. (12 N) HCl–ethanol–water 3:15:5]. The combined extract was dialysed against distilled water and freeze-dried. The resulting material was dissolved in 20 ml. of Gey buffer.

Statistics. Individual values for incorporation of radioactivity (counts/min.) or glucose uptake (mg. of glucose/g. of wet tissue/hr. for different pairs of hemidiaphragms incubated under identical conditions) were grouped, averaged and the standard error of the mean was determined. The significance of the difference between means was assessed on the basis of Student's *t* test.

RESULTS

Effect of plasma from alloxan-diabetic rats on the uptake of glucose and incorporation of amino acid by normal diaphragm

Two days after the subcutaneous administration of a diabetogenic dose of alloxan the plasma from these animals, unlike that of normal rats, possessed no detectable insulin-like activity when tested by its effect either on the uptake of glucose by isolated diaphragm from the normal rat, or on the incorporation of [^{14}C]glycine into protein of isolated diaphragm from the normal rat (Table 1). Freezing and thawing of the plasma three times exerted no significant effect on the results of such tests (Table 1).

Plasma from the 'long-term' alloxan-diabetic rat also had no observable effect on the uptake of glucose by isolated diaphragm from the normal rat, in contrast with plasma from the normal rat. This was true for untreated plasma and for plasma which had been frozen and thawed three times (Table 2). Untreated plasma nevertheless stimulated incorporation of [^{14}C]glycine into protein of isolated diaphragm from the normal rat, though to a much less extent than did plasma from the normal rat; this effect was not altered by freezing and thawing three times (Table 2).

These results suggest that plasma from either 'short-term' or 'long-term' starving diabetic rats contains little or no detectable insulin nor does it contain insulin, the activity of which is masked by the sort of insulin antagonist which is inactivated by freezing and thawing. To test this inference the extraction of insulin-like material by acid–ethanol was attempted. No insulin activity was found in material extracted thus from plasma of 'long-term'

Table 1. *Effect of blood plasma from 'short-term' alloxan-diabetic rats on the uptake of glucose and incorporation of [^{14}C]glycine into protein by the isolated diaphragm from the normal rat*

All the rats used to provide plasma were starved 24 hr. before being bled. Each result is the mean value, together with the standard error of the mean, for six experiments with six pairs of hemidiaphragms. The values of P for differences which appear possibly to be significant on inspection are indicated.

Expt. no.	Addition to the medium	No. of donor rats providing plasma	Glucose uptake (mg./g. wet wt./hr.)		Radioactivity in diaphragm protein (counts/min./disk)	
1	Plasma from normal rats	3	4·02±0·22	$P < 0·001$	396± 6	$P < 0·1 > 0·05$
	Buffer (control)	0	2·55±0·16		337±27	
	Plasma from diabetic rats*	6	2·38±0·24		289±27	
	Diabetic plasma frozen and thawed three times	6	2·21±0·11		305±23	
2	Buffer (control)	0	2·46±0·15		207± 7	
	Plasma from diabetic rats†	12	2·54±0·11		201±10	
	Diabetic plasma frozen and thawed three times	12	2·78±0·13		187±11	

* Mean fasting glucose content of pooled plasma from six alloxan-diabetic rats was 1080 mg./100 ml.
† Mean fasting glucose content of pooled plasma from twelve alloxan-diabetic rats was 885 mg./100 ml.

Table 2. *Effect of blood plasma from 'long-term' alloxan-diabetic rats on the uptake of glucose and incorporation of [^{14}C]glycine into protein by the isolated diaphragm from the normal rat*

All the rats used to provide plasma were starved 24 hr. before being bled. Each result is the mean value, together with the standard error of the mean, for six experiments with six pairs of hemidiaphragms. The values of P for differences which appear possibly to be significant on inspection are indicated.

Addition to the medium	No. of donor rats providing plasma	Glucose uptake (mg./g. wet wt./hr.)		Radioactivity in diaphragm protein (counts/min./disk)	
Plasma from normal rats	3	4·20±0·11	$P < 0·001$	318±6	$P < 0·001$
Buffer (control)	0	3·22±0·10		245±9	
Plasma from diabetic rats*	6	3·23±0·10		272±6	$P < 0·05$
Diabetic plasma frozen and thawed three times	6	3·22±0·09		280±9	$> 0·02$
					$P < 0·05 > 0·02$

* Blood-sugar values for the six alloxan-diabetic rats used as blood donors: fasting 102–340 mg./100 ml. (mean 171 mg./100 ml.); non-fasting 500–700 mg./100 ml.

Table 3. *Effect of material extracted by acid–ethanol from precipitated proteins of blood plasma from normal, 'short-term' and 'long-term' diabetic rats on the uptake of glucose and incorporation of [^{14}C]glycine into protein by isolated rat diaphragm from the normal rat*

All the rats used to provide plasma were starved 24 hr. before being bled. Each result is the mean value, together with the standard error of the mean, for six experiments with six pairs of hemidiaphragms. The values of P for differences which appear possibly to be significant on inspection are indicated.

Expt. no.	Addition to medium of protein extracted from plasma of	No. of donor rats providing plasma	Glucose uptake (mg./g. wet wt./hr.)		Radioactivity in diaphragm protein (counts/min./disk)	
1	Normal rats	3	3·84±0·15	$P < 0·001$	493±26	$P < 0·02 > 0·01$
	None (control with medium alone)	0	2·59±0·30	$P < 0·02 > 0·01$	407±12	$P < 0·1 > 0·05$
	Short-term diabetic rats*	6	3·68±0·19		458±23	
	Long-term diabetic rats†	6	2·64±0·18		389±14	
2	None (control with medium alone)	0	2·46±0·15		207±7	
	Short-term diabetic rats‡	12	2·75±0·22		212±8	

* Mean fasting glucose content of pooled plasma from six short-term alloxan-diabetic rats was 365 mg./100 ml.
† Mean fasting glucose content of pooled plasma from six long-term alloxan-diabetic rats was 472 mg./100 ml.
‡ Mean fasting glucose content of pooled plasma from twelve short-term alloxan-diabetic rats was 885 mg./100 ml.

Table 4. *Comparison of the incorporation of* [^{14}C]*glycine into protein, the uptake of glucose and the effect of insulin* (0·1 *unit/ml.*) *on these processes, by isolated diaphragm from the normal and the 'long-term' alloxan-diabetic rat*

Number of pairs of hemidiaphragms used is shown in parentheses. The values of P for differences which appear possibly to be significant on inspection are indicated. The effect of insulin is in every case significant ($P < 0{\cdot}001$).

Diaphragm from	Radioactivity in diaphragm protein (counts/min./disk)			Glucose uptake (mg./g. wet wt./hr.)		
	No insulin in medium	Insulin added	Difference	No insulin in medium	Insulin added	Difference
No glucose added to the medium						
Normal rats (6)	498±23	752±17	254			
Alloxan-diabetic rats (6)	399±29	675±46	276			
	$P < 0{\cdot}02 > 0{\cdot}01$					
Glucose added to the medium						
Normal rats (6)	490±26	798±69	308	3·04±0·16	6·01±0·32	2·97
Alloxan-diabetic rats (6)	406±21	676±41	270	2·45±0·31	5·82±0·34	3·37
	$P < 0{\cdot}05 > 0{\cdot}02$					
Pooled results						
Normal rats (12)	494±17	775±34	281			
Alloxan-diabetic rats (12)	402±17	676±29	274			
	$P < 0{\cdot}001$	$P < 0{\cdot}05 > 0{\cdot}02$				

alloxan-diabetic rats, in contrast with results with plasma from normal rats, either with respect to uptake of glucose or incorporation of [^{14}C]glycine into protein by isolated diaphragm from normal rats (Table 3). When one sample of blood plasma from 'short-term' alloxan-diabetic rats (mean fasting plasma glucose 365 mg./100 ml.) was treated thus, the extracted material effected a significant stimulation both of the uptake of glucose and of the incorporation of [^{14}C]glycine into protein by normal rat diaphragm, but with material from another sample (mean fasting plasma glucose 885 mg./100 ml.) no detectable insulin activity was found (Table 3).

Incorporation of [^{14}C]*glycine by diaphragm from alloxan-diabetic rats*

Incorporation of [^{14}C]glycine into protein of the isolated rat diaphragm of 'long-term' alloxan-diabetic rats, none of which had received insulin for at least 3 days, was less than that with diaphragm from normal rats, though the effect of insulin *in vitro* was normal (Table 4). The basal uptake of glucose by diaphragm from the 'long-term' alloxan-diabetic rat was slightly, though not significantly, less than by diaphragm from the normal rat, but in the presence of insulin the total glucose uptakes were similar (Table 4).

As might be expected, treatment of 'long-term' alloxan-diabetic rats with insulin (1 unit/day for 5 days) before removal of the diaphragm raised the rate of incorporation of [^{14}C]glycine into protein of the isolated diaphragm (Table 5). Administration of a single dose of insulin (1 unit) to the intact rat 30 min. before removal of the diaphragm also stimulated the incorporation of ^{14}C from a mixture of ^{14}C-labelled amino acids into protein of the isolated diaphragm (Table 6).

As judged by the fasting blood-sugar level our rats were acutely diabetic 48 hr. after treatment with alloxan. Rather surprisingly, however, incorporation of [^{14}C]glycine into protein of diaphragm from these animals was above normal, although the uptake of glucose was significantly less than that found with diaphragm from normal rats (Table 7). The results with respect to incorporation were similar in the presence or absence in the medium of glucose (Table 7). These results with 'short-term' alloxan-diabetic rats contrast greatly with those obtained with diaphragm from 'long-term' alloxan-diabetic rats (Table 4). With diaphragm removed only 12 hr. after the administration of alloxan the uptake of glucose and the incorporation of glycine *in vitro* were a little lower, though not significantly so, than with diaphragm from the normal rat (Table 8).

Administration of alloxan to rabbits brings about a rise in blood sugar which is succeeded by

hypoglycaemia (Jacobs, 1937; Hughes, Ware & Young, 1944). Hughes *et al.* (1944) concluded that the hypoglycaemic action of alloxan could result from the liberation of pre-formed insulin by necrotic pancreatic-islet cells. A hypersecretion of insulin in the early stages after administration of alloxan might explain the increased rate of incorporation of [^{14}C]glycine by diaphragm *in vitro* 48 hr. later. But if this were so, a rise and not a depression of glucose uptake would be expected, and a depression was in fact found (Table 7). However, any growth hormone secreted as a result, direct or indirect, of the administration of alloxan might depress the uptake of glucose by diaphragm (Park, Brown, Cornblath, Daughaday & Krahl, 1952) and stimulate incorporation of amino acids (Kostyo & Knobil, 1959; Manchester *et al.* 1959). It was therefore thought to be of interest to ascertain the effect of the 'short-term' administration of alloxan to the hypophysectomized rat on glycine incorporation by diaphragm *in vitro*. These experiments were also extended to include adrenalectomized animals.

Incorporation of [^{14}C]glycine into protein of isolated diaphragm from the hypophysectomized rat 48 hr. after injection of alloxan is greater than that found with diaphragm from the untreated hypophysectomized rat (Table 9), but incorporation into diaphragm from the adrenalectomized rat is not changed 48 hr. after administration of alloxan (Table 9). These results suggest that the enhanced rate of incorporation of [^{14}C]glycine into protein of diaphragm, observed 48 hr. after the administration of alloxan, is not the result of any change of activity of the pituitary gland, but is dependent upon the presence of the adrenal glands. Since the administration of cortisol to a rat results in a depression of the rate of the incorporation of labelled amino acids into the subsequently isolated diaphragm (Manchester *et al.* 1959; Wool & Weinshelbaum, 1959), it seems unlikely that the greater incorporation, which follows 48 hr. after treatment with alloxan, results from a rise in the secretion of corticosteroids. Injection of alloxan almost certainly elicits a secretion of adrenaline by the adrenal medulla, but neither the addition of adrenaline *in vitro* (Table 10), nor pre-treatment of the rat with adrenaline (Table 10), stimulates incorporation of [^{14}C]glycine into protein of isolated normal diaphragm.

Dr A. Korner (personal communication) finds that treatment of the rat with a large dose of cortisol (1 or 2 mg./day) depresses below normal the incorporation *in vitro* of labelled amino acids into protein by a microsomal system prepared from liver. Nevertheless, treatment of the rat with a small dose of cortisol (0·1 or 0·2 mg./day) stimulates incorporation in this system. The possibility therefore arises that in some circumstances adrenal corticoids may enhance the incorporation of labelled amino acids into protein of diaphragm, and that the stimulation of incorporation which follows the administration of alloxan may result from a small rise in the rate of secretion of adrenal corticoids. We have found no experimental evidence for this, however, since treatment of intact rats with cortisol, 0·1 mg. twice daily for 48 hr. before removal of the diaphragm, has no effect on the rate of incorporation of [^{14}C]-glycine into the protein of isolated diaphragm (Table 10). But the precise conditions for success in this respect may be highly critical.

Table 5. *Effect of treatment of the 'long-term' alloxan-diabetic rat with protamine–zinc insulin (1 unit/rat/day for 5 days) on the incorporation of [^{14}C]glycine into protein of the isolated diaphragm*

The medium contained glucose (2·5 mg./ml.). Number of pairs of hemidiaphragms used is shown in parentheses. The value of P for the difference is indicated.

Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)	
1% NaCl (9)	159±15	$P = 0{\cdot}02$
Insulin (8)	218±11	

DISCUSSION

The insulin content of the blood plasma of alloxan-diabetic rats

The observation that blood plasma from both 'short-term' and 'long-term' alloxan-diabetic rats, unlike plasma from normal rats, does not stimulate glucose uptake by isolated diaphragm from normal rats (Tables 1 and 2) suggests that these plasmas

Table 6. *Effect of treatment of the intact rat with insulin on the uptake of glucose, and on the incorporation of ^{14}C from a mixture of ^{14}C-labelled amino acids into protein, of the isolated diaphragm*

Each figure represents the mean result for six pairs of hemidiaphragms. The values of P for the differences are indicated. Insulin (1 unit/rat) was injected subcutaneously into the rat 30 min. before removal of the diaphragm.

Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)		Glucose uptake (mg./g. wet wt./hr.)	
1% NaCl	1276±67	$P < 0{\cdot}001$	2·37±0·26	$P < 0{\cdot}001$
Insulin	1908±66		5·60±0·49	

Table 7. *Comparison of the incorporation of* [^{14}C]*glycine into protein, the uptake of glucose, and the effect of insulin* (0·1 *unit/ml.*) *on these processes, by isolated diaphragm from the 'short-term' alloxan-diabetic rat*

Number of pairs of hemidiaphragms used is shown in parentheses. The values of P for differences which appear possibly to be significant on inspection are indicated. The effect of insulin is significant in every case ($P < 0·05$).

Diaphragm from	Radioactivity in diaphragm protein (counts/min./disk)			Glucose uptake (mg./g. wet wt./hr.)		
	No insulin in medium	Insulin added	Difference	No insulin in medium	Insulin added	Difference
No glucose added to the medium						
Normal rats (6)	241±17	395±23	154	—	—	—
Alloxan-diabetic rats (6)	336±25 } $P < 0·02 > 0·01$	450±42	114	—	—	—
Glucose added to the medium						
Normal rats (6)	303±9 } $P < 0·001$	446±18	143	2·78±0·05 } $P < 0·001$	6·21±0·15	3·43
Alloxan-diabetic rats (6)	467±33	592±20	125	2·15±0·09	5·57±0·24	3·42
Pooled results						
Normal rats (12)	272±8 } $P < 0·001$	421±14 } $P < 0·001$	149	—	—	—
Alloxan-diabetic rats (12)	401±21	521±22	120	—	—	—

contain much less insulin than does the plasma of normal rats. Moreover, no insulin-like material, extractable by acid–ethanol, could be detected in plasma of both 'short-term' and 'long-term' alloxan-diabetic rats with a high fasting blood-glucose level, either on the basis of stimulation of the uptake of glucose or on that of the incorporation of glycine into protein of isolated diaphragm from normal rats (Table 3). Nevertheless, such plasmas probably contain some insulin, since the depancreatized rat, completely deprived of insulin, survives less than 48 hr. (Folgia, 1944). Evidence for the presence of some insulin in the blood plasma of alloxan-diabetic rats is provided by the observation of the effect of their plasma on the incorporation of [^{14}C]glycine into protein of isolated diaphragm from normal rats, but although a significant stimulation of incorporation is produced by plasma from the 'long-term' alloxan-diabetic rat (Table 2), the magnitude of this stimulation is less than that produced by plasma from normal rats.

That no additional insulin activity appears when plasma is frozen and thawed three times (Tables 1 and 2) suggests that any putative antagonists to insulin, which might mask the presence of quantities of insulin that can be detected by use of the rat

Table 8. *Uptake of glucose and incorporation of* [^{14}C]*-glycine into protein by isolated rat diaphragm, 12 hr. after administration of alloxan to the rat*

Each figure represents the mean result for six pairs of hemidiaphragms. The differences are not significant.

Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)	Glucose uptake (mg./g. wet wt./hr.)
1% NaCl	248±12	2·16±0·17
Alloxan	226± 7	1·85±0·07

Table 9. *Incorporation of* [^{14}C]*glycine into protein of isolated diaphragm from hypophysectomized and adrenalectomized rats, 48 hr. after administration of alloxan*

The medium contained no added glucose. Number of pairs of hemidiaphragms used is shown in parentheses. The value of P for a difference which appeared to be possibly significant on inspection is indicated.

Animals	Radioactivity in diaphragm protein (counts/min./disk)	
Hypophysectomized rats treated with		
1% NaCl (6)	234± 9 }	$P < 0·05 > 0·02$
Alloxan (5)	282±17 }	
Adrenalectomized rats treated with		
1% NaCl (6)	311± 5	
Alloxan (6)	314±12	

Table 10. *Effect of adrenaline and of cortisol acetate on the incorporation of* [^{14}C]*glycine into protein of isolated rat diaphragm*

Each figure represents the mean result for six pairs of hemidiaphragms. The difference between means is not significant.

Expt. no.	Addition to the medium	Rats treated with	Radioactivity in diaphragm protein (counts/min./disk)	Glucose uptake (mg./g. wet wt./hr.)
1	No addition	—	258±8*	—
	Adrenaline†	—	251±9	—
2	No addition	1% NaCl	258±11*	1·82±0·09
	No addition	Adrenaline‡	231±13	1·91±0·12
3	No addition	1% NaCl	283±7§	—
	No addition	Cortisol acetate‖	288±10	—

* Medium contained glucose (2·5 mg./ml.). † (1 μg./ml.).
‡ Adrenaline (0·05 mg.) subcutaneously 1 hr. before removal of the diaphragm.
§ Medium contained no added glucose. ‖ 0·1 mg./rat twice daily for 2 days.

diaphragm *in vitro*, are not inactivated by freezing and thawing. Any such antagonists must differ from the inhibitor which appears in the blood of the cat when the animal is treated with growth hormone (Randle & Young, 1956) and are unlikely to be lipoproteins of the sort described by Bornstein (1953). Since Krahl, Tidball & Bregman (1959) have shown that a lipoprotein fraction, obtained from the plasma of normal rats, inhibits the stimulation by insulin of the uptake of glucose by normal rat diaphragm as effectively as does lipoprotein from the serum of the alloxan-diabetic rat, there is no reason to suppose that the lipoprotein inhibitor is peculiar to the alloxan-diabetic rat.

It seems more likely that the alloxan-diabetic rat, after the first 24 hr., is suffering from an acute lack of secreted insulin rather than from a masking of the biological activity of circulating insulin by an insulin antagonist. Ash, Pennington & Reid (1959) found that the administration of butyrate raised the blood-sugar level of the insulin-treated sheep to a much greater extent than did administration of an equal amount of glucose. Such an observation suggests that fatty or keto acids may antagonize the action of insulin. During the first few days after the administration of alloxan the diabetic rat, if not protected with insulin, loses reserve fat and suffers from a ketosis which can be fatal. It may be that antagonism of the little remaining insulin by circulating fatty and keto acids explains the large number of fatalities during the first week after administration of alloxan and the insulin resistance encountered during this phase. When the body-fat reserves have been considerably depleted the danger of ketosis is lessened.

Effect of induction of alloxan-diabetes on the behaviour of rat diaphragm in vitro

The induction of alloxan diabetes is known to decrease the uptake of glucose by rat diaphragm examined *in vitro* (Krahl & Cori, 1947; Villee & Hastings, 1949). Krahl & Cori (1947) concluded that the rate of glucose uptake *in vitro* by diaphragm from alloxan-diabetic rats is depressed only when the fasting blood-sugar level is above 300 mg./100 ml. Our results are in accordance with these observations. With 'short-term' starving alloxan-diabetic rats (in which the fasting blood-sugar level is high) the uptake of glucose by diaphragm *in vitro* is much below that for diaphragm from normal rats (Table 7), whereas the uptake of glucose by diaphragm from starving 'long-term' alloxan-diabetic rats (in which the average fasting blood-sugar level is not very high) was not so clearly sub-normal (Table 4). It may be, however, that the decreased glucose uptake of diaphragm from rats with a blood-sugar level in excess of 300 mg./100 ml. is more apparent than real. Bornstein & Park (1953) found that under the conditions normally employed for pre-treatment of diaphragm in which the tissue is soaked in cold buffer for 5–10 min. it is unlikely that glucose, already present in the diaphragm, will diffuse from the tissue into the buffer. Diaphragm from hyperglycaemic rats will, therefore, carry over more glucose into the final incubation medium than will diaphragm from normal rats and this glucose will be released into the medium during incubation with shaking at 37°, giving the impression of a smaller disappearance of glucose from the medium than is really the case.

Krahl (1953) examined the incorporation *in vitro*, in a medium to which no glucose was added, of [^{14}C]glycine into glutathione and protein of liver slices, and into protein of isolated diaphragm, with starving alloxan-diabetic rats 7 days after treatment with alloxan. In these experiments incorporation was below that found for tissues for normal rats, but was raised almost to the normal value by the addition of glucose alone to the medium. The fasting blood-sugar levels of these rats ranged from 110 to 438 mg./100 ml., but no correlation was observed for individual rats between the height of the blood

sugar and the rate of incorporation *in vitro*. In agreement with some of these results of Krahl (1953) we find that incorporation of [^{14}C]glycine into protein of diaphragm from the starving 'long-term' alloxan-diabetic rat is below normal (Table 4). We also find no correlation between the blood sugar of the diaphragm donors and the incorporation of glycine. From our experience of the chronic alloxan-diabetic rat we consider that the fasting blood sugar is not a reliable or adequate measure of the intensity of the diabetes, as judged by the degree of emaciation of individual rats and the ability of their isolated diaphragms to incorporate [^{14}C]glycine. As with diaphragm from normal rats, addition of glucose to the medium does not stimulate incorporation by diaphragm from 'long-term' alloxan-diabetic animals (Manchester, 1960). [See Manchester (1960) for a discussion of the possible reasons for these results differing from those of Krahl (1953).]

The high rate of amino acid incorporation and low glucose uptake of isolated diaphragm, observed 48 hr. after the administration of alloxan to the intact rat, is difficult to explain. The high rate of incorporation could result from an excessive liberation of insulin from necrotic pancreatic-islet cells (Hughes *et al.* 1944) but the effect of any such insulin ought also to be seen on glucose uptake, and its presence in the plasma should be detectable by an effect on normal rat diaphragm. This action of alloxan is abolished by removal of the adrenal glands, but no evidence has been found to suggest that the secretion of either glucocorticoids or adrenaline is responsible for the stimulation of glycine incorporation, though it is possible that we have not achieved the right conditions for success in this respect. There remains the possibility that administration of alloxan leads to the release of androgenic material from the adrenals and that this material produces a stimulation of amino acid incorporation into protein in diaphragm. We have not as yet been able to provide experimental support for this possibility, although it has certainly not been excluded.

SUMMARY

1. The effect of different treatments of the rat on the metabolism of its diaphragm, removed and examined *in vitro*, have been studied with respect to the uptake of glucose, the incorporation of [^{14}C]-glycine into protein, and the effect of the addition *in vitro* of insulin.

2. Two days after the administration of a diabetogenic dose of alloxan the fasting blood sugar of rats was usually about 500 mg./100 ml. Blood plasma from such animals contained no detectable insulin. The incorporation of amino acid *in vitro* by diaphragm from these alloxan-treated rats was above normal.

3. Two weeks or more after diabetes-inducing treatment with alloxan the fasting blood sugar averaged 215 mg./100 ml. Plasma from these animals had no observable effect on the glucose uptake by isolated diaphragm from the normal rat. Untreated plasma and plasma which had been frozen and thawed stimulated incorporation of [^{14}C]glycine into protein of diaphragm from the normal rat, though to a less extent than did plasma from the normal rat.

4. Two days after the administration of alloxan to the intact or to the hypophysectomized rat incorporation of [^{14}C]glycine into protein of diaphragm was significantly greater than that with diaphragm from animals not treated with alloxan.

5. Two days after the administration of alloxan to the adrenalectomized rat incorporation of [^{14}C]-glycine into protein of the animal's diaphragm *in vitro* was similar to that with diaphragm from the untreated adrenalectomized rat. Treatment of the intact rat with adrenaline or with cortisol failed to mimic the effect of the administration of alloxan in stimulating the incorporation of [^{14}C]glycine *in vitro* into protein of isolated diaphragm.

6. With diaphragm from rats which had received diabetes-inducing treatment with alloxan two or more weeks previously, the basal uptake of glucose and the basal incorporation of [^{14}C]glycine were both slightly less than those for diaphragm from the normal rat, but the effect of insulin *in vitro* on these processes was normal. Treatment of the diabetic rat with insulin for 5 days raised the basal incorporation of glycine *in vitro* to about the value for diaphragm from the intact rat.

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Studies on the Chemical Basis of the Antigenicity of Proteins

3. THE ROLE OF RIGIDITY IN THE ANTIGENICITY OF POLYPEPTIDYL GELATINS*

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An investigation of the antigenicity of various polypeptidyl gelatins (Sela & Arnon, 1960*a*) showed that the enrichment with tyrosine, tryptophan or phenylalanine converted gelatin into relatively powerful antigens of narrow specificity. The attachment of cysteine to gelatin caused a limited enhancement of antigenicity. Enrichment of gelatin with alanine, lysine or glutamic acid did not increase significantly the antigenic response.

From a comparison of the antigenic specificity of several polytyrosyl gelatins (Arnon & Sela, 1960) it was concluded that the serological specificity changes strongly as a function of the tyrosine content. Thus gelatin cross-precipitated with antibodies to a derivative with a low tyrosine content, inhibited the homologous reaction of a material with a somewhat higher tyrosine content, but did not interfere at all with the serological reaction of a derivative enriched with the largest amount of tyrosine (11%). It seemed of interest to investigate the cross-reaction of the various polypeptidyl gelatins with the antiserum to gelatin. Here, again, the increase in the tyrosine content diminished considerably the extent of precipitation.

In view of the above-mentioned results we assumed that the enhancement of the antigenicity of gelatin by the attachment of the aromatic α-amino acids and of cysteine was due to an increase in the rigidity of the molecule. According to Haurowitz (1952) a rigid structure of the determinant groups is a prerequisite for antigenicity. Even though the content of sulphydryl groups in polycysteinyl gelatin did not change significantly during a period of several months, it is possible that disulphide bonds, which would increase the rigidity of the macromolecule, are formed *in vivo*. It was therefore of interest to investigate the antigenicity of polyseryl gelatin. Serine differs from cysteine only by the oxygen which replaces sulphur, and thus no cross-linking bridges may be formed as a result of the attachment of serine to gelatin. As expected, the attachment of serine did not convert gelatin into a more powerful antigen.

In order to elucidate the role of the aromatic character of the aromatic amino acids attached, in the enhancement of the antigenicity, we have now investigated the immunological activity of poly-*cyclo*hexylalanyl gelatins. This paper presents evidence that the attachment of amino acids containing the fully saturated *cyclo*hexane rings caused a strong increase in the antigenicity, as compared with the original gelatin.

* Part 2: Arnon & Sela (1960).

It seems thus that areas of rigidity are a necessary prerequisite for the enhancement of the antigenicity of gelatin. The question arises: what are the rigid sites responsible for the antigenicity of the unmodified gelatin molecule? Gelatin contains very few aromatic acids. It has a high percentage of glycine, which interferes with the stability of an α-helical structure. On the other hand, the high content of proline and hydroxyproline in this heat-denatured protein might still contribute towards helical areas of the type found in collagen (Ramachandran & Kartha, 1954; Rich & Crick, 1955) and in poly-L-proline (Cowan & McGavin, 1955; Harrington & Sela, 1958; Steinberg, Harrington, Berger, Sela & Katchalski, 1960). We have therefore attempted to inhibit the specific gelatin–antigelatin precipitin reaction by means of various copolymers containing proline or hydroxyproline or both. The polymers tested had no influence on the homologous precipitin reaction.

EXPERIMENTAL

Materials

The names of amino acids are abbreviated as in Brand & Edsall (1947) and in Sanger (1952) (see Sela & Arnon, 1960*a*). *cyclo*Hexylalanine is abbreviated as Cha. The samples of gelatin, egg albumin, the polypeptidyl gelatins pAlaGel, pGluGel, pLysGel, pPheGel and pCysGel, the polytyrosyl egg albumin, as well as a copolymer of L-glutamic acid and L-phenylalanine in a residue molar ratio of 1:1, were described by Sela & Arnon (1960*a*). The polytyrosyl gelatins (pTyrGelA, pTyrGelB, pTyrGelC) were described by Arnon & Sela (1960).

Poly-L-serine [number-average degree of polymerization (*n* 30)] was a gift from Mr Y. Lapidot. Poly-L-proline (*n* 170) was obtained from Mr I. Z. Steinberg. Poly-L-hydroxyproline (*n* 95), a copolymer of L-proline and glycine (in a residue molar ratio of 1:3, *n* 30), a copolymer of L-proline and sarcosine (in a residue molar ratio of 1:1, *n* 50) and a copolymer of L-proline, L-hydroxyproline and glycine (in a residue molar ratio of 1:1:1, *n* 60) were gifts from Dr J. Kurtz. The amino acid sequence in the above copolymers is random. A copolymer of L-ProGlyGly (obtained by the polymerization of a tripeptide derivative, number-average molecular weight 1200) was a gift from Mr Y. Wolman. A copolymer of L-proline, L-glutamic acid and glycine (in a residue molar ratio of 1:1:2) was prepared by the copolymerization in pyridine of *N*-carboxy-L-proline anhydride, γ-benzyl-*N*-carboxy-L-glutamate anhydride and *N*-carboxyglycine anhydride, followed by debenzylation of the polymerization product with anhydrous hydrogen bromide.

Polypeptidyl proteins

*Poly-*L*-cyclohexylalanyl gelatin A* (pChaGelA), *poly-*L*-cyclohexylalanyl gelatin B* (pChaGelB) *and poly-*L*-cyclohexylalanyl egg albumin.* These were prepared, analogously to other polypeptidyl gelatins and egg albumins (Sela, 1954; Sela & Arnon, 1960*a*; Arnon & Sela, 1960), by the reaction of *N*-carboxy-L-*cyclo*hexylalanine anhydride with gelatin (Sela & Arnon, 1960*b*). pChaGelA contained 4·4% of *cyclo*hexylalanine residues. pChaGelB contained 10·9% of *cyclo*hexylalanine residues. Analysis of the dinitrophenylated pChaGelB showed that in this substance 23 moles of distinct poly-L-*cyclo*hexylalanine chains were attached on the average per 100 000 g. of gelatin (Sela & Arnon, 1960*b*). The average number of *cyclo*hexylalanine residues per peptide chain was, in this case, 3·5. Poly-L-*cyclo*hexylalanyl egg albumin contained 9·7% of *cyclo*hexylalanine residues. A polypeptidyl gelatin enriched with both L-glutamic acid and L-*cyclo*hexylalanine (pGluChaGel) was also prepared (Sela & Arnon, 1960*b*). It contained 19·3% of glutamic acid residues (original gelatin contains 10·2% of glutamic acid residues) and 18·3% of *cyclo*hexylalanine residues. A copolymer of L-*cyclo*hexylalanine and L-glutamic acid, in a residue molar ratio of 1:1 (*n* 50), was prepared by the copolymerization of *N*-carboxy-L-*cyclo*hexylalanine anhydride and γ-benzyl-*N*-carboxy-L-glutamate anhydride, followed by the debenzylation of the polymerization product with anhydrous hydrogen bromide in acetic acid (Sela & Arnon, 1960*b*).

*Poly-*L*-prolyl gelatin* (pProGel). This was prepared by adding 2·5 m-moles of *N*-carboxy-L-proline anhydride (Kurtz, Fasman, Berger & Katchalski, 1958) in dioxan/g. of gelatin in 0·05 M-phosphate buffer, pH 7, according to the method of synthesis of polypeptidyl gelatins (Sela & Arnon, 1960*a*). The water-soluble reaction product was dialysed against distilled water at 2° for 3 days and freeze-dried. The material contained 25·3% of proline residues, determined by the method of Troll & Lindsley (1955) as modified by Sarid, Berger & Katchalski (1959) (the original gelatin contained 13·1% of proline residues).

*Poly-*L*-seryl gelatin* (pSerGelA). This compound was prepared by adding 5 m-moles of *O*-acetyl-*N*-carboxy-L-serine anhydride (J. Kurtz, unpublished work)/g. of gelatin. After 3 days at 2° the reaction mixture was brought to pH 13 with 2 M-sodium hydroxide and maintained for 2 hr. at room temperature. The clear solution was dialysed against distilled water at 2° for 3 days and freeze-dried. The material contained 10·2% of serine residues, as determined by ninhydrin colorimetry (Kay, Harris & Entenman, 1956) after chromatography in pyridine–water (65:35, v/v) of the acid hydrolysate, as well as 0·6% of acetyl groups. Commercial bone gelatin contains 3·1% of serine residues (Eastoe, 1955).

Methods

Immunization procedure. The antigens used were incorporated in a water-in-oil adjuvant mixture, as described by Sela & Arnon (1960*a*). Ready prepared adjuvants were obtained from Difco Laboratories, Detroit 1, Mich., U.S.A. Each material was injected into two rabbits. After pre-immunization bleedings, the antigen was injected into the thighs of the hind-legs of the animals. Four injections of 0·5 ml. of the adjuvant mixture were given, at intervals of 14 days. The animals were bled weekly by cardiac puncture, 2–5 weeks after the last injection. Thiomersal was added to all sera to a concentration of 0·01%.

Quantitative precipitin studies. No precipitation occurred when any of the materials tested were added to the pre-immunization sera. In preliminary tests qualitative precipitin reactions were carried out on antisera of individual rabbits. In all experiments the antisera to a particular

antigen behaved similarly. Thereafter such antisera were pooled before quantitative studies. The quantitative precipitin tests were performed by the usual techniques; when no precipitation occurred, the more refined technique of Maurer (1954) was used. Both methods have been described by Sela & Arnon (1960*a*).

Inhibition studies. Substances checked as possible inhibitors of the precipitin reactions were added at various concentrations, in a volume of 0·1 ml., to 0·2 ml. of serum. After an incubation of 30 min. at 37° an amount of antigen corresponding to the optimum precipitation was added, and the mixture was incubated at 37° for 1 hr. and then placed in the cold room for 24 hr.

Hydroxyproline. This was determined in the hydrolysates (6 N-hydrochloric acid, 105°, 24 hr.) of the antigen–antibody precipitates, as well as in the hydrolysates of the gelatin derivatives themselves, by the method of Neuman & Logan (1950). Commercial bone gelatin contains 11·5% of hydroxyproline residues (Eastoe, 1955).

Spectrophotometric measurements. These were made on a Beckman model DU spectrophotometer, at approx. 25°, with quartz cells of 1 cm. light path, with a capacity of 1 ml.

RESULTS

*Poly*cyclo*hexylalanyl gelatins*

Two poly-L-*cyclo*hexylalanyl gelatins, containing 4·4 and 10·9% of *cyclo*hexylalanine residues respectively, were tested for an immunological response in rabbits. No precipitation occurred when solutions of these materials were added to pre-immunization sera. The antisera were subjected to the precipitin test. Positive reactions were obtained in both cases. Table 1 gives the analysis of the precipitates from the antisera tested with the homologous antigens. The amount of antigen in the precipitates was determined from the hydroxyproline content of the acid hydrolysates. As *cyclo*hexylalanine does not absorb light in the ultraviolet region, the antibody content of the precipitates was obtained directly from the extinction of the neutralized solutions at 280 mμ.

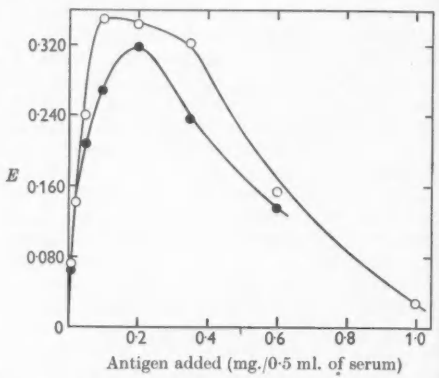

Fig. 1. Extinction of neutral solutions of precipitates obtained by the addition of poly-L-*cyclo*hexylalanyl gelatin A (○) and poly-L-*cyclo*hexylalanyl gelatin B (●) to the antiserum to poly-L-*cyclo*hexylalanyl gelatin A, as a function of the amount of precipitant.

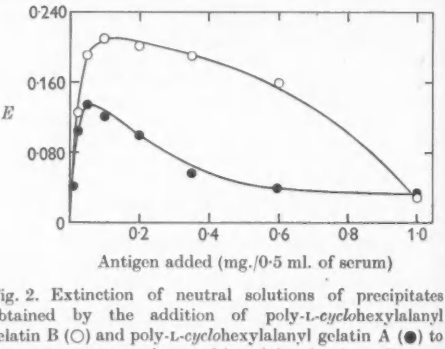

Fig. 2. Extinction of neutral solutions of precipitates obtained by the addition of poly-L-*cyclo*hexylalanyl gelatin B (○) and poly-L-*cyclo*hexylalanyl gelatin A (●) to the antiserum to poly-L-*cyclo*hexylalanyl gelatin B, as a function of the amount of precipitant.

Table 1. *Composition of precipitates of the systems pChaGelA–anti-pChaGelA and pChaGelB–anti-pChaGelB*

Antigen	Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/antigen in precipitate (w/w)
pChaGelA	20	22	100	115	5·2
	50	52	100	224	4·3
	100	72	72	378	5·2
	200	95	47·5	550	5·8
	400	138	34·5	540	3·9
	700	164	23·4	502	3·1
	1200	87	7·3	246	2·8
	2000	31	1·6	41	1·3
pChaGelB	50	49·5	100	196	4·0
	100	74·5	74·5	296	4·0
	200	106·6	53·3	330	3·1
	400	180	45	316	1·8
	700	184	26·2	298	1·6
	1200	252	21	250	1·2

The antibodies to the two poly*cyclo*hexylalanyl gelatins are very specific. They could be precipitated by both antigens. The precipitin curves of the homologous and cross-reactions are given in Figs. 1 and 2. Precipitation was obtained also when poly-L-*cyclo*hexylalanyl egg albumin was added to the antisera. No cross-precipitation occurred upon addition of any of the materials listed as possible inhibitors in Table 2, as well as unrelated proteins such as egg albumin, to the antisera.

All those substances that did not precipitate anti-pChaGelA or anti-pChaGelB were tested for their ability to inhibit the specific precipitin reactions. Table 2 summarizes the inhibition experiments. Complete inhibition was observed in both systems only with a gelatin derivative enriched with both *cyclo*hexylalanine and glutamic acid. Gelatin enriched with phenylalanine (pPheGel) is a better inhibitor of the pChaGelB–anti-pChaGelB than of the pChaGelA–anti-pChaGelA reaction. The increased reactivity of pPheGel with antibodies to the derivative richer in *cyclo*hexylalanine points to some steric similarity between the two ring structures. As both gelatin and a linear copolymer containing L-*cyclo*hexylalanine are effective inhibitors of the two systems, it may be concluded that the active antigenic sites include both *cyclo*hexylalanine and parts of the unmodified gelatin molecule.

Table 2. *Inhibition tests*

In all cases 20, 100 and 1000 μg. of the inhibiting substance was added to 0·2 ml. of each antiserum, 30 min. before the addition of the homologous antigen. −, Complete inhibition at the highest inhibitor concentration, inhibitory influence even at the lowest concentration; +, partial inhibition at the highest concentration, no effect at the lowest concentration; + +, only slight inhibition at the highest concentration; + + +, no inhibitory effect.

Inhibitor	Effect on pChaGelA system	Effect on pChaGelB system
pGluChaGel	−	−
pPheGel	+ +	−
pTyrGelA	+ + +	+ + +
pTyrGelC	+ +	+ +
Gelatin	+	+
Copolymer *cyclo*hexylalanine Glu (1:1)	+	+
Copolymer Phe Glu (1:1)	+ +	+ +

Polyseryl gelatin

Antiserum against poly-L-seryl gelatin (pSerGelA) gave no precipitate with the homologous antigen, when subjected to the usual tests. Nor was any precipitation observed when the antiserum was caused to cross-react with gelatin and various polypeptidyl gelatins. The precipitin test was therefore performed in a way similar to that used by Maurer (1954) to demonstrate the antigenicity of unmodified gelatin. Table 3 gives the analyses of the precipitates from the antiserum tested with the homologous antigen.

Antibodies to pSerGelA were precipitated under the above-described conditions also with gelatin, pCysGel and pTyrGelA (Fig. 3). pCysGel was selected because of the chemical similarity between cysteine and serine, and pTyrGelA was tested as a typical unrelated modified gelatin. Poly-L-serine neither precipitated nor inhibited the homologous precipitin reaction.

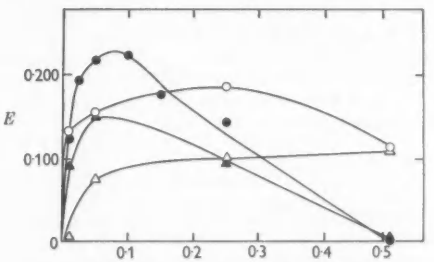

Fig. 3. Extinction of neutral solutions of precipitates obtained by the addition of poly-L-seryl gelatin (●), poly-L-cysteinyl gelatin (○), gelatin (▲) and poly-L-tyrosyl gelatin A (△) to the antiserum to poly-L-seryl gelatin, as a function of the amount of precipitant.

Table 3. *Composition of precipitates of the system poly-L-seryl gelatin–anti-poly-L-seryl gelatin*

Precipitin reaction according to Maurer (1954).

Antigen added (μg./ml. of serum)	Antigen precipitated (μg.)	Antigen precipitated (%)	Antibody precipitated (μg.)	Antibody/antigen in precipitate (w/w)
5	5·1	100	58	10·5
12	12·2	100	91	7·5
20	14·0	70	103	7·3
40	15·8	39·5	107	6·8
60	14·6	24·3	83·5	5·7
100	14·6	14·6	67·5	4·6
400	6·2	1·5	0	0

Cross-precipitation and inhibition of the gelatin–antigelatin reaction

Antiserum to gelatin was obtained by immunization of three rabbits. The maximal amount of precipitate (118 μg. of antibody/1 ml. of serum) was obtained by the technique of Maurer (1954) by adding 150 μg. of the antigen to 4·5 ml. of the antiserum.

The results of precipitation of antibodies to gelatin with various polypeptidyl gelatins are given in Table 4. The extent of cross-precipitation decreases with the increase in the amount of amino acids attached to gelatin in the derivatives checked. Thus pTyrGelC, enriched with only 2·4% of tyrosine residues, is almost as good a precipitant as the homologous antigen, whereas pTyrGelA, enriched with 11·1% of tyrosine residues, causes a much smaller precipitation. The gelatin derivatives of L-tryptophan, L-lysine and L-glutamic acid, which cross-precipitated only to a limited extent, were enriched with more than 20% of the amino acid residues attached.

The following amino acid polymers and copolymers were tested for their possible inhibitory effect on the gelatin–antigelatin system: poly-L-proline; poly-L-hydroxyproline; random copolymers of L-proline and glycine; L-proline and sarcosine; L-proline, L-hydroxyproline and glycine; L-proline, L-glutamic acid and glycine; a low-molecular-weight polymer of the tripeptide ProGlyGly. None of the above substances had any influence on the homologous precipitin reaction.

DISCUSSION

The experiments described demonstrate that the enrichment of gelatin with an α-amino acid containing the *cyclo*hexane ring in its side chain converts it into a relatively powerful antigen. The amounts of antibodies precipitated compare favourably with those obtained by the precipitation of antibodies to polytyrosyl gelatins with the homologous antigens. It may be concluded that the previously observed (Sela & Arnon, 1960*a*) strong enhancement of the antigenicity of gelatin by attachment of aromatic amino acids is not due to their aromatic character. The common denominator for the two sets of results seems to be, rather, the increase in the rigidity of the antigen molecule. Although the *cyclo*hexane ring is not as rigid as the aromatic benzene ring, it is known that certain energetically favoured configurations may become the stable ones. The chair conformation of *cyclo*hexane is favoured energetically over that of a boat by approximately 6 kcal. For methyl*cyclo*hexane an equatorial conformation is more stable than the axial one by 1·8 kcal. (Beckett, Pitzer & Spitzer, 1947; Newman, 1956).

The attachment of serine to gelatin did not increase, in contrast to the attachment of cysteine, the extent of antigenicity. The serine derivative cannot form intramolecular bridges. On the other hand, a cysteine derivative readily forms such links. This is, again, in agreement with the suggested role of rigidity in the antigenicity of a molecule.

From the investigation of the cross-reaction of various polypeptidyl gelatins with the antiserum to gelatin it may be deduced that the attachment of peptide chains to gelatin interferes with the reactivity of the specific antigenic sites.

The use of synthetic polypeptides in inhibition experiments, intended to detect the chemical nature of the antigenically active sites of gelatin, was unsuccessful. As the precipitation of anti-gelatin with gelatin may be prevented by an adequate inhibitor, such as a chymotryptic digest

Table 4. *Cross-precipitation of antiserum to gelatin with various polypeptidyl gelatins*

Extinction of neutralized solutions of the precipitates obtained upon addition of the precipitants to 1 ml. of the antiserum.

	Amount of precipitant added (μg./ml. of serum)					
Precipitant	7	11	27	33	67	110
Gelatin	0·089	0·142	0·181	0·178	0·171	0·087
pAlaGel	—	0·083	—	0·120	—	0·045
pCysGel	—	0·157	—	0·157	—	0·122
pGluGel	—	0·029	—	0·067	—	0·040
pLysGel	—	0·051	—	0·073	—	0·065
pPheGel	—	0·058	—	0·102	—	0·164
pProGel	—	0·130	—	0·142	—	0·070
pSerGelA	—	0·164	—	0·118	—	0·013
pTryGel*	—	0·052	—	0·070	—	0·123
pTyrGelA*	0·022	—	0·040	—	0·045	0·032
pTyrGelB*	0·087	—	0·154	—	0·148	—
pTyrGelC*	0·104	—	0·156	—	0·148	—

* Extinction is not corrected for the extinction of the precipitants.

of gelatin (Maurer, 1958), it may be concluded that the active antigenic site has a chemical structure much more intricate than that present in the various copolymers tested by us.

The sequence GlyProHypro is assumed to be of frequent occurrence in collagen and gelatin (Schroeder, Kay, LeGette, Honnen & Green, 1954; Rich & Crick, 1955). If the areas of rigidity responsible for the antigenicity of the gelatin molecule are due to its proline and hydroxyproline content, it is possible that a high-molecular-weight copolymer containing repeating sequences of GlyProHypro, when available, might inhibit the specific serological reaction.

SUMMARY

1. Poly-L-*cyclo*hexylalanyl gelatins are relatively powerful antigens, as followed by the precipitin reaction. The antibodies to the two antigens investigated could be also cross-precipitated by other poly*cyclo*hexylalanyl derivatives.

2. The attachment of serine did not convert gelatin into a more powerful antigen. Antibodies to poly-L-seryl gelatin could not be detected by the usual precipitin techniques, but their presence could be shown by a method similar to that used by Maurer (1954) to demonstrate the antigenicity of gelatin.

3. In view of the increase in antigenicity as a result of the attachment of aromatic amino acids, *cyclo*hexylalanine or cysteine to gelatin, it was concluded that areas of rigidity in the molecule are a necessary prerequisite for the enhancement of the antigenicity of gelatin.

4. The use of some synthetic polypeptides containing proline or hydroxyproline or both in inhibition experiments, intended to detect the chemical nature of the antigenically active sites of gelatin, was unsuccessful.

5. Various polypeptidyl gelatins were caused to cross-react with the antiserum to gelatin. An increase in the amount of the amino acid attached to gelatin diminished considerably the extent of precipitation.

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A Method for the Quantitative Fractionation of Mixtures of 2-Methoxyoestrone, Oestrone, Ring D α-Ketolic Oestrogens, Oestradiol-17β, 16-*epi*Oestriol and Oestriol by Partition Chromatography and the Girard Reaction

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The object of this investigation was to develop a method for the quantitative fractionation of pure 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol. This required, first, a technique for the Girard reaction which would result in quantitative recoveries, and, secondly, a method whereby the ketonic and non-ketonic oestrogen residues could be separated into fractions by partition chromatography.

EXPERIMENTAL

Materials

Reagents used were A.R. unless otherwise stated. Diethyl ether, benzene, methanol, ethanol (96%, by wt.), Celite 535 and the Kober reagents were purified, prepared and tested as described by Bauld & Greenway (1957). The final distillation of solvents was done in all-glass apparatus.

Absolute ethanol. Purified ethanol (96%, by wt.) was left overnight with calcium oxide (300 g./l.), refluxed for 1 hr. and distilled under anhydrous conditions. Magnesium ethoxide was prepared with 75 ml. of dry ethanol, 5 g. of magnesium turnings and 0·5 g. of iodine. Dry ethanol (925 ml.) was then added, refluxed for 4 hr. and distilled (Vogel, 1951).

Hexane. Hexane (Anachemia, Montreal, Canada, technical grade, b.p. 65–69°) was washed with conc. H_2SO_4 (6 × 85 ml./l.) for 45 min. per washing, water (2 × 350 ml./l.), N-$KMnO_4$ in 3·7 N-H_2SO_4 (4 × 85 ml./l.) and left overnight with the last $KMnO_4$ portion. The hexane was then washed with water (4 × 170 ml./l.), 0·5 M-Na_2CO_3 (1 × 35 ml./l.), water (4 × 170 ml./l.) and dried over Na_2SO_4. The solvent (up to 3 l.) was poured through a column (48 in. × 1 in.) of 6–12-mesh silica gel, distilled and the 67·2–68·8° fraction collected.

Stained glassware was cleaned with a solution of 0·4 N-oxalic acid in 3·7 N-H_2SO_4.

Girard P *reagent* (*Girard & Sandulesco*, 1936). Commercial material was refluxed with acid-washed charcoal in ethanol (96%) for 15 min., filtered and left overnight at 5°. The crystals were washed with ethanol and stored in a brown bottle in a desiccator.

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Asbestos. The material (Anachemia, Montreal, Canada, long fibre) was stirred with an excess of conc. HCl, washed with water until the washings were neutral and free of Cl^- and Fe^{3+} ions and dried at 140° for 24 hr.

Reference compounds. 16-*epi*Oestriol was obtained through the courtesy of Professor G. F. Marrian, 16-oxo-oestradiol-17β from Professor Marrian and Dr M. Levitz and 2-methoxyoestrone from Dr T. F. Gallagher. 16α-Hydroxyoestrone was prepared in collaboration with our colleagues Dr R. Greenway and Dr D. Douglas by a method based on that of Leeds, Fukushima & Gallagher (1954). The optical rotation of the prepared 16α-hydroxyoestrone agreed with the value reported by Marrian, Loke, Watson & Panattoni (1957). As judged by column chromatography (for details see below) and paper chromatography (Givner, Bauld & Vagi, 1960), the eight steroids used in these studies were pure.

Fractionation and measurement of oestrogens

The final procedure (Fig. 1) is described below.

Separation of the ketonic from the non-ketonic oestrogens by the Girard reaction. The procedure for the Girard reaction was a modification of the method described by Pope, McNaughton & Jones (1957) and by Marrian, Watson & Panattoni (1957).

A mixture of about 10 μg. of each oestrogen in ethanol was warmed and the solvent evaporated *in vacuo*. The residue was dried under $CaCl_2$ for 4 hr. in a vacuum desiccator. Absolute ethanol (1·26 ml.), Girard P reagent (0·126 g.) and acetic acid (0·25 ml.) were added to the flask. The reaction flask was left at room temperature (21 ± 3°) for 17 hr. and then cooled in a refrigerator (5°) for 1 hr. This was followed by the addition of 15 ml. of ice-water and sufficient 2·5 N-NaOH (cold reagent, approx. 1·5 ml.) to give a solution of pH 6. Three extractions were made with 20 ml. portions of ether, leaving the aqueous phase containing the ketonic fraction. The time for the separation of the two mixed phases was about 4 min. The ether extracts were combined and washed with 5 ml. of ice–water, and the aqueous phase was combined with the rest of the ketonic fraction. The ether extracts were washed with 2·5 ml. of M-$NaHCO_3$ (separation time of the phases, 7 min.), which was followed by a 5 ml. rinsing of the separating funnel, a wash (5 ml.) and a rinsing (20 ml.), all with water. The pH of the final rinsing was checked for neutrality (pH paper, range pH 2–10; Fisher Scientific Co., Montreal, Canada). The term 'wash' is used to describe the procedure that involved inversions of the separating funnel for the equilibration of the aqueous and organic phases;

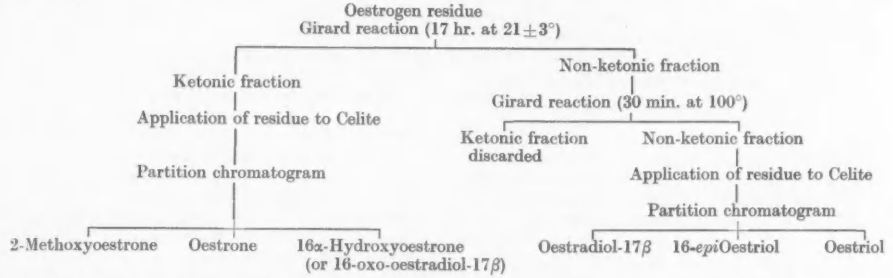

Fig. 1. Flow sheet of the method for the separation of 2-methoxyoestrone, oestrone, 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β), oestradiol-17β, 16-*epi*oestriol and oestriol.

a rinsing describes the addition of water for the removal of alkali from the inside walls of the separating funnel. The ether was distilled off, leaving the non-ketonic fraction. A second Girard reaction was carried out on the non-ketonic fraction to ensure removal of trace amounts of ketones not previously separated. The reaction was carried out by refluxing (side stem protected with $CaCl_2$) the non-ketonic fraction for 30 min. on a boiling-water bath with the same quantities of reagents as described above. After extraction of the non-ketonic fraction, the aqueous phase was discarded.

The aqueous (ketonic) fraction of the first Girard reaction was acidified to 1·2 N with 7 N-H_2SO_4 (approx. 4·5 ml.), left at room temperature for 2 hr. and extracted with ether (3 × 30 ml.). The ether was washed with 4 ml. of M-$NaHCO_3$ by 300 inversions of the separating funnel. This was followed by a rinsing (5 ml.), a wash (5 ml., pH checked for neutrality) and two rinsings (20 and 10 ml., after the final rinsing, pH was checked for neutrality), all with water. The extract was dried over anhydrous Na_2SO_4 and the ether distilled off, leaving the ketonic fraction.

Preparation of chromatographic column. Solvents were equilibrated and the chromatography was performed at a temperature of 18±0·5°. The separated aqueous (stationary) phase was added to dry Celite (5 ml./5 g.) and stirred for 3 min. An excess of organic (mobile) phase was added to the damp Celite and a uniform slurry was obtained by thorough mixing. A small amount of asbestos fibre (approx. 20 mg.) was inserted at the bottom of a chromatographic column (1 cm. × 15 cm.) to act as a filter plug to trap Celite particles that might have been eluted with the oestrogen. The column was supported with the lower end below the surface of a few millilitres of mobile phase in a test tube. Small portions of slurry were added to the top of the column and packed to the required height (10 or 12 cm.) by means of a plunger having a 0·71 mm. hole in the disk (Bauld, 1955). Sufficient pressure was exerted during the packing to give a solvent flow rate of 16–18 ml./hr.

Determination of column parameters. The periodic check of column parameters was necessary to make certain that no inert, mobile or stationary phase was lost during the packing of the columns and to ensure their reproducibility. The determination of column parameters was based on the work of Martin & Synge (1941) and Butt, Morris, Morris & Williams (1951). The equation of Butt *et al.* (1951) may be rearranged to give

$$V_r = KLA_s + LA_L, \quad (1)$$

where V_r = the retention volume = volume of eluate to peak concentration of solute (ml.); K = partition coefficient = (concentration of solute in stationary phase)/(concentration of solute in mobile phase); L = length of column (cm.); A_s = cross-sectional area (cm.²) of column occupied by stationary phase; A_L = cross-sectional area (cm.²) of column occupied by mobile phase. For these determinations a slurry containing a known weight of Celite was packed in the column and the length of the column measured. The weight of the dry Celite (W_I) used and its density (D_I, 2·34) was used to calculate the volume of the inert phase (V_I, equation 2). From V_I and the length of the column, the cross-sectional area of the inert phase (A_I, equation 3) was calculated:

$$V_I = \frac{W_I}{D_I}, \quad (2)$$

$$A_I = \frac{V_I}{L}. \quad (3)$$

The cross-sectional area of the mobile phase (A_L) was determined from L and the retention volume of a dye (Sudan Red) with zero partition coefficient for the stationary phase. The dye was applied to the column in mobile phase and the eluate from the column collected until the dye appeared at its maximum concentration in the eluate. This volume was then the retention volume (V_r) of a solute of $K = 0$. From equation (1), when $K = 0$,

$$A_L = \frac{V_r}{L}. \quad (4)$$

The cross-sectional area of the empty column (A cm.²) was determined from the volume of water occupied in a known length of column

$$A = \frac{V}{L}. \quad (5)$$

The cross-sectional area of the stationary phase (A_s) was calculated by the difference between the cross-sectional area of the empty column and the sum of A_I and A_L:

$$A_s = A - (A_I + A_L). \quad (6)$$

The theoretical A_s was calculated from the volume of stationary phase (V_s) added to the Celite and the length of the column:

$$A_s = \frac{V_s}{L}. \quad (7)$$

The experimental A_s should agree within limits of experimental error with the theoretical A_s; if significantly lower, stationary phase is being lost from the column. Furthermore, to maintain constant elution patterns of the chromatograms, the A_s to A_L ratio should be reproducible from column to column.

Application of solute to column. The low solubility of 16α-hydroxyoestrone and oestriol in hexane saturated with methanol–water (70:30, v/v) made quantitative transfers to a column impractical by the method of Bauld (1955) and required a procedure similar to that of Lemieux & Charanduk (1951). Celite (0·2–0·3 g.) was added to a test tube ($\frac{5}{8}$ in. × $2\frac{1}{8}$ in.) and compressed into a pad with the plunger. The test tube was heated to 76±4° by a dry heating apparatus such as the electric heater in Fig. 2. The oestrogen residue in the flask was dissolved in 1 ml. of methanol and transferred in small amounts (approx. 2 × 0·5 ml.) to the top surface of the Celite pad with care to avoid wetting the sides of the test tube; the complete procedure was repeated twice. The successive transfers (approx. 0·5 ml.) were carried out only when the previous portion was evaporated. After the final transfer and evaporation, the Celite pad was broken up into particles with a glass rod and reheated for 5 min. The tube was stored in a closed container at 5° and warmed to chromatography temperature about 1 hr. before use. The contents of the tube were transferred to the top of the column through a funnel with three rinsings of mobile phase. Dry Celite (50–100 mg.) was added to the column and compressed with a plunger. In column parameter studies, the eluate appearing during the application of solute was included in the determination of the retention volumes of the oestrogen or dye.

Partition chromatography of the non-ketonic fractions. The non-ketonic oestrogen fraction was transferred to the Celite pad as previously described. The chromatogram was prepared with the following solvent system: stationary phase, methanol–water (70:30, v/v); mobile phase no. 1, benzene–hexane (55:45, v/v); mobile phase no. 2, benzene.

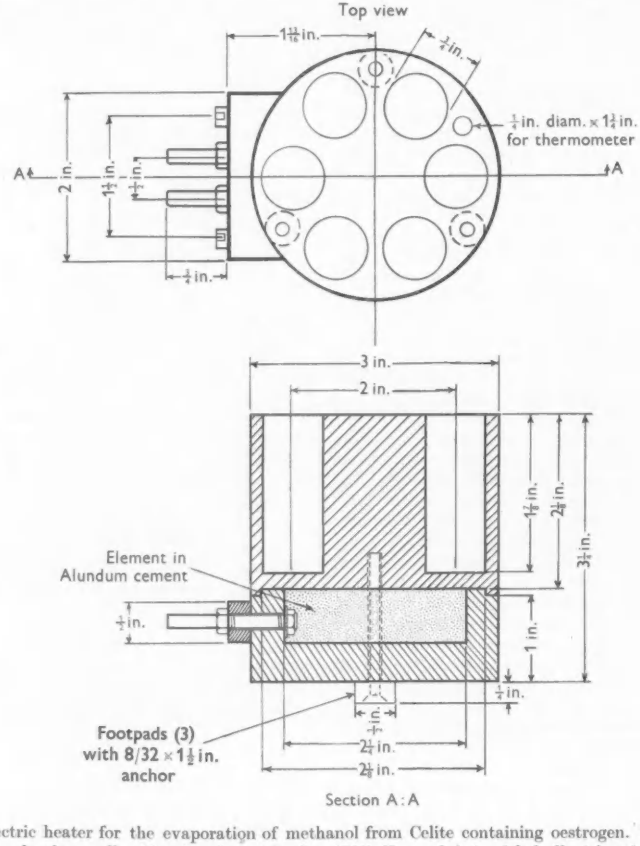

Fig. 2. An electric heater for the evaporation of methanol from Celite containing oestrogen. The heater was made from an aluminum alloy Dural and contained a 150Ω Chromel A (a nickel alloy) heating element that was connected to a voltage control set at 30 v a.c.

Table 1. *Recoveries of oestrogens submitted to the Girard reaction by the final procedure*

Oestrogen added	Amount added (μg.)	No. of expts.	Reaction time (hr.)	Reaction temp.	Recovery (%) (non-ketonic) Mean	Recovery (%) (non-ketonic) Range	Recovery (%) (ketonic) Mean	Recovery (%) (ketonic) Range
2-Methoxyoestrone	9·8	8	17	21±3°	0	0	99	95–100
Oestrone	10·1	8	17	21±3	3	2–4	96	94–99
16α-Hydroxyoestrone	11·9	4	17	21±3	9	9–10	88	86–91
16-Oxo-oestradiol-17β	10·0	4	17	21±3	3	2–3	93	93–94
Oestradiol-17β	10·0	19	17	21±3	100	94–107	0	0
Oestradiol-17β	10·0	4	0·5	130±3	99	98–100	0	0
16-*epi*Oestriol	9·2	2	17	21±3	100	100	0	0
16-*epi*Oestriol	9·2	4	0·5	130±3	100	100	0	0
Oestriol	9·9	4	17	21±3	100	98–100	0	0
Oestriol	9·9	8	0·5	130±3	97	94–101	0	0

The column was packed from a slurry containing 5 ml. of stationary phase (equilibrated with mobile phase no. 1) and 5 g. of Celite; after stirring, excess of mobile phase no. 1 was added to the slurry. The Celite-column dimensions were 1 cm. × 10 cm. The extract was transferred to the column. Rinsing of the test tube containing the extract was done with mobile phase no. 1 from a separating funnel (containing 60 ml.), which was then fitted to the top of the column. The funnel had a stopper with an air leak. The eluate from 0 to 10 ml. was discarded and the next 50 ml. collected, giving the oestradiol-17β fraction. With mobile phase no. 2, the 16-*epi*oestriol and oestriol fractions were eluted with 45 and 107 ml. respectively. The solvents were removed *in vacuo* by heating in a boiling-water bath.

Partition chromatography of the ketonic fraction. The ketonic oestrogen fraction was transferred to the Celite pad as previously described. The chromatogram was prepared with the following solvent system: stationary phase, methanol–water (70:30, v/v); mobile phase no. 1, hexane–benzene (98:2, v/v); mobile phase no. 2, hexane–benzene (50:50, v/v). The column was packed from a slurry containing 5 ml. of stationary phase (equilibrated with mobile phase no. 1) and 5 g. of Celite; after stirring, excess of mobile phase no. 1 was added to the slurry. The Celite column dimensions were 1 cm. × 12 cm. The extract was transferred to the column. Rinsing of the test tube which contained the extract was done with mobile phase no. 1 from a separating funnel (containing 52 ml.) having a stopper and air leak. The eluate from 0 to 10 ml. was discarded and the next 42 ml. was collected, giving the 2-methoxyoestrone fraction. With mobile phase no. 2, the oestrone and 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β) fractions were eluted with 40 and 92 ml. respectively. The solvents were removed *in vacuo* by heating in a boiling-water bath.

Colorimetric estimation. The oestrogen residues were dissolved in ethanol and portions were transferred to test tubes (23 mm. × 150 mm.); quinol (25±5 mg.) was added and the solvent evaporated in a boiling-water bath with an air stream. The Kober reaction was carried out by the procedure previously described for three oestrogens (Bauld, 1956). 'Oestradiol' reagent (3·3 ml.) was added to the oestradiol-17β and 2-methoxyoestrone residues; 'oestrone' reagent (3·0 ml.) was added to the oestrone residue; 'oestriol' reagent (2·6 ml.) was added to the 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β), 16-*epi*oestriol and oestriol residues. The tubes were then heated for 20 min. in a boiling-water bath (shaking after 5 and 10 min.), cooled in cold water and quinol was added (25±5 mg.). Water (0·3 ml.) was added to the test tube containing oestrone and 0·7 ml. to those containing the 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β), 16-*epi*-oestriol and oestriol residues. The tubes were reheated for 15 min. (shaking after 5 and 10 min.), cooled in cold water and extinctions were measured against reagent blanks in a Beckman model B spectrophotometer. Readings were corrected for non-specific pigments by applying Allen correction equations (Allen, 1950). For oestrone, 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β), 16-*epi*-oestriol and oestriol the wavelengths were 480, 512·5 and 545 mμ; for oestradiol-17β they were 480, 515 and 550 mμ; for 2-methoxyoestrone they were 512·5, 547·5 and 582·5 mμ. Appropriate standard curves were prepared. The amount of oestrogen in each fraction was calculated from its corrected extinction and that of the appropriate standard by simple proportion.

RESULTS

Experimental conditions and recoveries on seven oestrogens submitted to the Girard reaction

Many preliminary experiments were necessary to determine and establish the experimental conditions for the Girard reaction used in the final procedure. The recoveries by the final technique are shown in Table 1. The salient results of the preliminary studies on the individual oestrogens are described below.

2-Methoxyoestrone. The oestrogen was partly destroyed when in water (12% loss after 1 hr.), in 4N-HCl (21%) and in 4N-H_2SO_4 (26%). The destruction by the 4N-H_2SO_4 increased with time. However, the oestrogen was not affected by treatment with 1·2N-H_2SO_4 for 1 hr. but was partially destroyed by 1·2N-HCl (18%). The addition of 36N-H_2SO_4 (final solution was 1·2N) for the hydrolysis of the Girard complex partly destroyed the oestrogen (12–18%), but this was not true of 7N-H_2SO_4 (final solution was 1·2N). With 7N-H_2SO_4, 2 hr. was required for the complete hydrolysis of the Girard complex.

Oestrone. Experiments showed that 3% of the oestrogen may remain in the non-ketonic fraction.

16α-Hydroxyoestrone. Studies showed that the 16α-hydroxyoestrone found in the non-ketonic fraction was not decreased by an increase in the reaction time from 17 to 24 hr. or a decrease in the amount of acetic acid. A change in pH from 6 to 7 after neutralization resulted in no improvement. The use of aq. NH_3 soln. instead of NaOH diminished the amount of oestrogen in the non-ketonic fraction but no significant increase was apparent in the ketonic fraction. When a second Girard reaction was carried out on the ketonic fraction, a small percentage of the oestrogen was found once more in the non-ketonic fraction. The non-ketonic fraction from a Girard reaction on 119 μg. of 16α-hydroxyoestrone was identified as 16α-hydroxyoestrone by paper chromatography.

16-Oxo-oestradiol-17β. Experiments showed that 93 % of this oestrogen was recovered in the ketonic fraction and 3 % in the non-ketonic fraction.

Oestradiol-17β, 16-epioestriol and oestriol. These oestrogens were quantitatively recovered with either a long reaction time at room temperature or a short reaction time at a high temperature (130°). The use of absolute ethanol appeared to be important for quantitative recoveries of oestriol.

Elution pattern of non-ketonic oestrogens

Fig. 3 shows the separation of oestradiol-17β, 16-*epi*oestriol and oestriol. In this experiment 28 ml. of eluate between the oestradiol-17β and 16-*epi*oestriol fractions, and 64 ml. between the 16-*epi*oestriol and oestriol fractions, contained no oestrogen. This system showed excellent reproducibility, as determined by the measurement of column parameters (Sudan Red was used as the dye with zero partition coefficient for the stationary phase). Typical column parameters were as follows: column length, 11·5 cm.; column area, 0·88 cm.²; inert-phase cross-sectional area, 0·15 cm.²; mobile-phase cross-sectional area, 0·37 cm.²; stationary-phase cross-sectional area, 0·36 cm.²; retention volume, 4·3 ml.

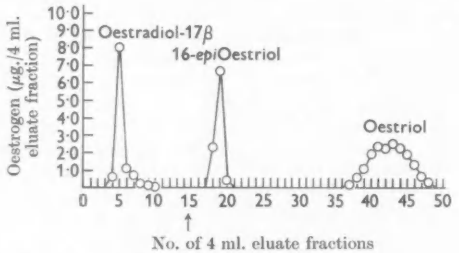

Fig. 3. Partition-column chromatography of oestradiol-17β, 16-*epi*oestriol and oestriol. Inert phase, Celite; stationary phase, methanol–water (70:30, v/v); mobile phase no. 1, benzene–hexane (55:45, v/v); mobile phase no. 2 (↑), benzene.

Elution pattern of ketonic oestrogens

Fig. 4 shows the separation of 2-methoxyoestrone, oestrone and 16α-hydroxyoestrone. In this experiment, 28 ml. of eluate between the 2-methoxyoestrone and oestrone fractions, and 48 ml. of eluate between the oestrone and 16α-hydroxyoestrone fractions, were free of oestrogen. Under similar experimental conditions 16-oxo-oestrone was eluted with oestrone and 16-oxo-oestradiol-17β with 16α-hydroxyoestrone. This system showed excellent reproducibility as determined by the measurement of column parameters (as described above, Sudan Red was used). Typical column parameters were as follows: column

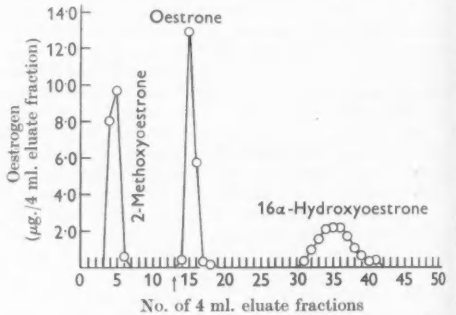

Fig. 4. Partition-column chromatography of 2-methoxyoestrone, oestrone and 16α-hydroxyoestrone. Inert phase, Celite; stationary phase, methanol–water (70:30, v/v); mobile phase no. 1, hexane–benzene (98:2, v/v); mobile phase no. 2 (↑), hexane–benzene (50:50, v/v).

Table 2. *Recovery of oestrogens added to Celite and chromatographed on columns*

Oestrogen added	Amount added (μg.)	No. of expts.	Percentage recovery (mean)
2-Methoxyoestrone	51	1	99
	20	1	90
Oestrone	67	2	94
	51	1	100
	20	1	97
	10	2	95
16α-Hydroxyoestrone	37	1	85
	15	1	89
16-Oxo-oestradiol-17β	16	1	94
Oestradiol-17β	24	2	96
	12	3	92
	10	3	95
16-*epi*Oestriol	47	1	91
	9	2	94
Oestriol	25	1	93
	20	1	87

length, 11·6 cm.; column area, 0·88 cm.²; inert-phase cross-sectional area, 0·15 cm.²; mobile-phase cross-sectional area, 0·35 cm.²; stationary-phase cross-sectional area, 0·38 cm.²; retention volume, 4·1 ml.

Recovery of oestrogens added to Celite and chromatographed on columns

Table 2 shows the satisfactory recoveries of pure oestrogens after they were applied to Celite, transferred to a partition-chromatography column and eluted.

DISCUSSION

The experiments on the Girard reaction indicate that under appropriate conditions the ketonic and the non-ketonic oestrogens may be separated quantitatively and without destruction. There has been disagreement about the quantitative value of this procedure in oestrogen methodology. Westerfeld, Thayer, MacCorquodale & Doisy (1938) reported that 70–90 % of added oestrone may be recovered in the ketonic fraction after the reaction. Pincus & Pearlman (1942) used the reaction successfully in the separation of urinary oestrone and oestradiol-17β. Levitz, Spitzer & Twombly (1956) carried out the original procedure of Girard & Sandulesco (1936) on 16-[16-^{14}C]-oxo-oestradiol-17β and found radioactivity in the non-ketonic fraction. Marrian (1957) discovered that 16-oxo-oestradiol-17β and 16α-hydroxyoestrone are both unstable in the Girard reaction in hot solution, but stable when it is performed at room temperature. The latter finding has been confirmed by the present investigation. In addition, we have found that a small amount of the 16α-hydroxyoestrone was in the non-ketonic fraction, although the Girard reaction was carried out at room temperature. Dr W. Klyne (personal communication) has suggested that the 16α-hydroxyl group may hinder the formation of the Girard complex at the 17-position. Other possible explanations include the formation of a water-insoluble azine (Bates, 1952) or the hydrolysis of the Girard complex during neutralization with alkali. In addition, our studies demonstrate that the Girard reaction can be used to separate 2-methoxyoestrone and oestrone satisfactorily from the non-ketonic oestrogens.

The partition columns and method of residue transfer described in this report differ from those of Bauld (1955); they have three distinct advantages. First, it is now possible to separate and measure six instead of three oestrogens. Secondly, the technique of residue transfer avoids the difficulty of transferring polar steroids in non-polar solvents. Thirdly, the high percolation rate is a valuable time-saver.

Recently, this method as developed for pure steroids has been incorporated into procedures for the separation and measurement of urinary oestrogens (Givner, Bauld & Vagi, 1960) and tissue oestrogens (Miss J. Mitchell and Dr R. Hobkirk, personal communication).

SUMMARY

1. A new chemical method which separates and quantitatively measures pure 2-methoxyoestrone, oestrone, 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β), oestradiol-17β, 16-*epi*oestriol and oestriol is described.

2. Experimental conditions for the Girard reaction resulting in quantitative recoveries of these oestrogens have been developed.

3. A technique for the quantitative transfer of absorbed oestrogen on Celite to a partition chromatogram is described.

4. Partition columns for the separation of 2-methoxyoestrone, oestrone, 16α-hydroxyoestrone (or 16-oxo-oestradiol-17β) and for the separation of oestradiol-17β, 16-*epi*oestriol and oestriol are described.

5. The possible applications of this method are discussed.

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A Chemical Method for the Quantitative Determination of 2-Methoxyoestrone, Oestrone, Ring D α-Ketolic Oestrogens, Oestradiol-17β, 16-*epi*Oestriol and Oestriol in Human Urine

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The object of this investigation was to develop a method for the quantitative determination of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol in human urine. The development of the method began with a detailed study of the experimental conditions required for quantitative separation of pure ketonic and non-ketonic oestrogens by means of the Girard reaction and the development of partition columns for the separation of the oestrogens in each of the Girard fractions (Givner, 1959; Givner, Bauld & Vagi, 1960).

The technique described in this report involves enzymic hydrolysis of the urine, ether extraction, separation of the ketonic from the non-ketonic oestrogens by the Girard reaction, the separation of the individual oestrogens by column-partition chromatography on Celite and colorimetric estimation. It differs from the methods of Brown (1955) and of Bauld (1956), which measure three urinary oestrogens, and from the method of Stimmel (1958), which separates five oestrogens by countercurrent distribution.

Extensive specificity studies on the urinary-oestrogen fractions are also described. In addition, the method is evaluated for accuracy, adaptability and sources of error.

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METHODS

Purification of materials. Diethyl ether, benzene, hexane, ethanol, methanol, Kober reagents, Girard reagent P, Celite and asbestos were purified as described by Givner *et al.* (1960). Glusulase, a *Helix pomatia* extract (Endo Laboratories, New York) and bacterial β-glucuronidase (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used as received. Blue tetrazolium reagent (dianisole-bis-4:4′-(3:5-diphenyl)tetrazolium chloride was recrystallized from ethanol (96 %, by wt.) by the addition of diethyl ether. pH papers (ranges pH 2–10 and pH 9–11) were purchased from Fisher Scientific Co., Montreal. Glassware was cleaned as previously described (Bauld, 1956).

Collection of urine. Urine samples were collected for 24 hr. without preservative and stored at 5° if necessary.

Fractionation of urine

The purification procedure for urine is described below; letter references are to the flow sheet of Fig. 1.

Hydrolysis of late-pregnancy urine (A). A 50 ml. (approx.) portion of a 24 hr. urine sample was adjusted to pH 5·2 with 0·1 N-acetic acid or 0·1 N-NaOH and then hydrolysed with Glusulase (500 Fishman units of β-glucuronidase/ml. of urine) for 24 hr. at 39 ± 0·5°. The units were as defined by the supplier and were based on the assay procedure of Talalay, Fishman & Huggins (1946). The urine was cooled to 5° and two 20 ml. portions of the hydrolysed urine were transferred to separating funnels, each containing 80 ml. of water.

Hydrolysis of male urine (A). A 24 hr. urine sample was diluted to 2·5 l. with water; a 250 ml. portion (approx.) was adjusted to pH 6·5, and hydrolysed with bacterial β-glucuronidase (Sigma Chemical Co.), 20 units/ml. of urine for 48 hr. at 37 ± 0·5°, and then cooled to 5°. The

(A) Urine hydrolysed with Glusulase or bacterial β-glucuronidase

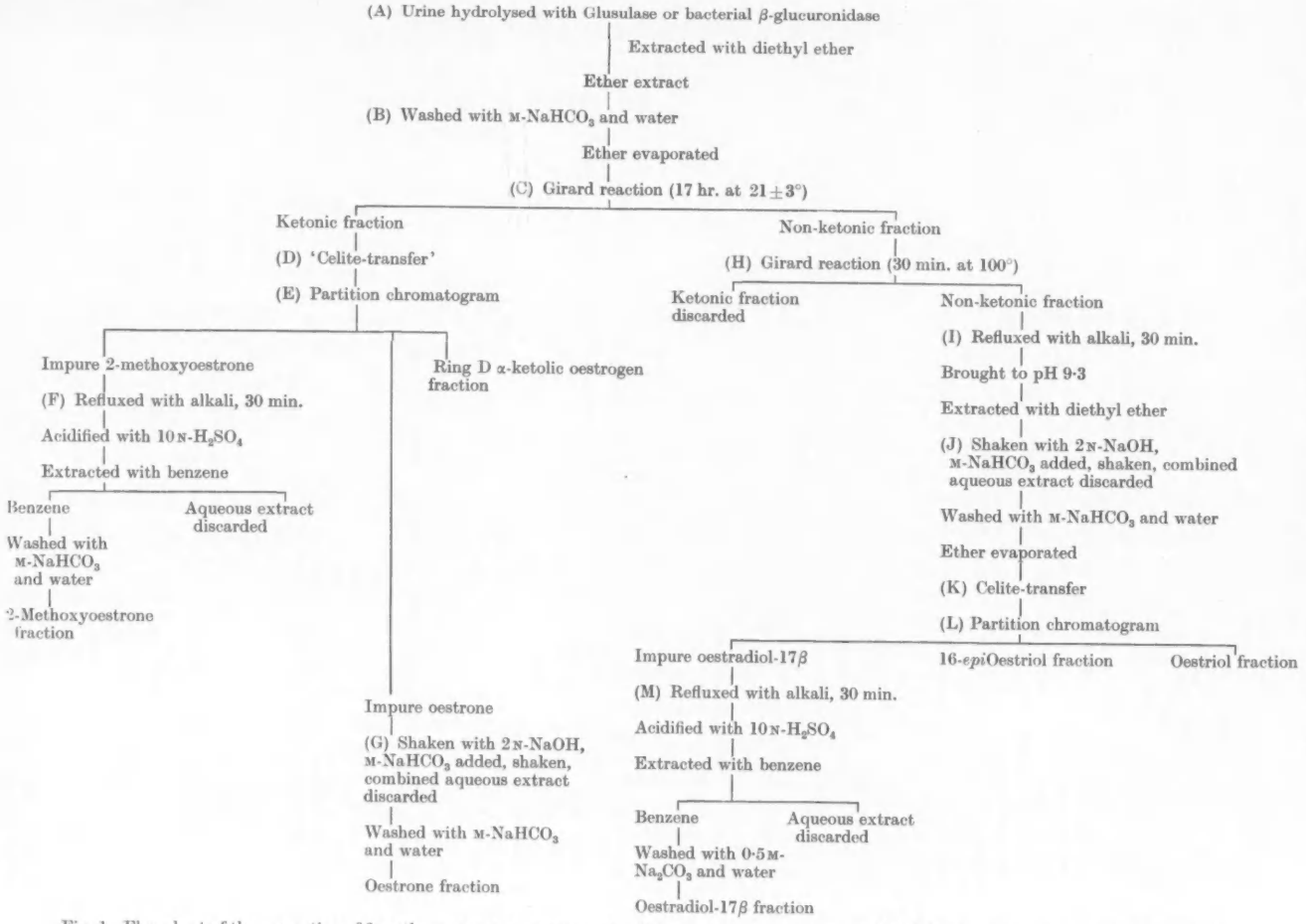

Fig. 1. Flow sheet of the separation of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol from urine.

units were as defined by the supplier and were essentially Fishman units except for small changes in the assay procedure (Sigma Chemical Co. 1951). Two 100 ml. portions of the hydrolysed urine were transferred to separating funnels and extracted. For certain male urines, a larger portion of hydrolysed urine was necessary for analysis and the quantities of reagents used in the extraction procedure were adjusted accordingly.

Extraction (*B*). The 100 ml. portion of dilute urine was extracted with ether (4 × 30 ml.). The ether extract was washed with 12 ml. of M-$NaHCO_3$, washed and rinsed with water (3 ml. for each procedure) to neutrality (pH paper, range pH 2–10), dried over anhydrous Na_2SO_4 and distilled to dryness on a boiling-water bath. The term 'wash' is used here to describe the procedure that involved inversions of the separating funnel for the equilibration of the aqueous and organic phases, whereas a rinsing describes the addition of water for the removal of alkali from the inside walls of the separating funnel. The flask containing the extract was connected to a water-suction pump (1–25 mm. Hg) for 5 min. at room temp. (21 ± 3°) to bring about partial removal of volatile impurities. The residue was transferred to a 100 ml. round-bottomed flask with ethanol (3 × 3 ml.), which was then removed *in vacuo*.

Girard reaction (*C*) *and* (*H*). The extract was dried over anhydrous $CaCl_2$ for 4 hr. in a vacuum desiccator and the reaction was carried out at room temperature. A second Girard reaction was carried out at an elevated temperature on the non-ketonic fraction. The experimental conditions for both procedures have been described previously (Givner *et al.* 1960).

Purification of non-ketonic fraction (*I*) *and* (*J*). This fraction was boiled on an electric heater under reflux for 30 min. with 82 ml. of N-NaOH. The solution was cooled, transferred to a separating funnel and brought to pH 9·3–9·5 (pH paper, range pH 9–11) by passage of CO_2. The solution was extracted with ether (4 × 40 ml.), the ether extracts were combined (J) and shaken with 2 N-NaOH (7·6 ml.). The aqueous phase, without removal from the separating funnel, was partially neutralized with M-$NaHCO_3$ (30·4 ml.); the ether and aqueous phases were again shaken. If the pH of the lower phase was 10 or less it was discarded; if the pH was higher than 10, more M-$NaHCO_3$ was added and the phases were again shaken. The ether phase was washed with M-$NaHCO_3$ (7·6 ml.), water (5 ml.) and distilled to dryness.

Partition chromatography of the non-ketonic fraction (*K*) *and* (*L*). The non-ketonic fraction was transferred to a Celite pad and the chromatogram was prepared as previously described (Givner *et al.* 1960). The oestradiol-17β, 16-*epi*oestriol and oestriol fractions were eluted and the solvents were removed *in vacuo*.

Partition chromatography of the ketonic fractions (*D*) *and* (*E*). The ketonic fraction was transferred to the Celite pad and the chromatogram was prepared as previously described (Givner *et al.* 1960). The 2-methoxyoestrone, oestrone and ring D α-ketolic oestrogen fractions were eluted and the solvents were removed *in vacuo*.

Purification of the oestradiol-17β fraction (*M*). This fraction was boiled under reflux for 30 min. with N-NaOH (10 ml.), acidified with 10 N-H_2SO_4 (1·1 ml.) and extracted with benzene (25 ml.). The extract was washed with 0·5 M-Na_2CO_3 (4 ml.) and water (4 ml.). This was followed by a rinsing with water (4 ml.). The benzene extract was transferred to a test tube containing quinol (25 ± 5 mg.) and the solvent removed by heating in a boiling-water bath in a stream of filtered air.

Purification of the 2-methoxyoestrone fraction (*F*). This was purified by the procedure described above for the oestradiol-17β fraction with one difference, namely the substitution of M-$NaHCO_3$ (4 ml.) for 0·5 M-Na_2CO_3 (4 ml.).

Purification of the oestrone fraction (*G*). The extract was transferred to a separating funnel with ether (4 × 40 ml.) and the ether extract was shaken with 2 N-NaOH (7·6 ml.). The aqueous phase, without removal from the separating funnel, was partially neutralized with M-$NaHCO_3$ (30·4 ml.) and the phases were shaken again. If the pH of the lower phase was 10 or less, it was discarded; however, if the pH was higher than 10, more M-$NaHCO_3$ was added and the phases were again shaken. The ether extract was washed with M-$NaHCO_3$ (7·6 ml.) and water (5 ml.). The ether solution was distilled to dryness.

Colour development on purified extracts. The oestrogen extracts (with the exception of the oestradiol-17β and 2-methoxyoestrone extracts, which were already in test tubes) were dissolved in ethanol and portions were transferred to test tubes; quinol (25 ± 5 mg.) was added and the solvent evaporated by heating in a boiling-water bath. The Kober reaction was carried out by the procedure described by Givner *et al.* (1960). Standard curves were prepared with six pure oestrogens (2-methoxyoestrone, 0–10 μg.; oestrone, 0–10 μg.; 16α-hydroxyoestrone, 0–12 μg.; oestradiol-17β, 0–10 μg.; 16-*epi*oestriol, 0–9 μg.; oestriol, 0–10 μg.). However, the amount of each oestrogen in each fraction was calculated from its corrected extinction and that of the appropriate standard by simple proportion. The 16α-hydroxyoestrone standard was used as the reference compound for the ring D α-ketolic oestrogen fraction.

Special procedures for specificity studies

In the identification of the column-chromatography eluates, paper chromatography was used. All systems were maintained at a constant temperature of 32 ± 1° as described by Mitchell & Davies (1954). The lid was sealed with starch paste (Zaffaroni, Burton & Keutman, 1951). The solvent systems were equilibrated overnight in the cabinet and the tank was equilibrated for 5 hr. Chromatography was performed on Whatman no. 1 filter paper washed as described by Axelrod (1954). Samples, standards of pure oestrogens and a solvent blank were applied to parallel strips (2 cm. × 46 cm.) by means of ethanol (3 × 0·05 ml.) and the solvent was evaporated by a stream of air. The diameters of the starting spots were 0·5 cm. (approx.). The strips were equilibrated for 1 hr. before the run was started. Detection of spots was by means of Folin & Ciocalteu's (1927) phenol reagent; elution was with ethanol by an apparatus described by Axelrod (1954). The solvent was evaporated *in vacuo*. For paper chromatography, methanol–water (70:30, v/v) was employed as the stationary phase with the following mobile phases: hexane–benzene (90:10, v/v) for 2-methoxyoestrone and oestrone fractions; hexane–benzene (80:20, v/v) for ring D α-ketolic fraction; hexane–benzene (95:5, v/v) for the oestradiol-17β fraction; hexane–benzene (50:50, v/v) for the 16-*epi*oestriol fraction; hexane–benzene (20:80, v/v) for the oestriol fraction.

For additional specificity studies, the six urinary-oestrogen fractions were prepared as described previously

and the absorption and fluorescence spectra measured (Bauld, Givner, Engel & Goldzieher, 1960). For ultraviolet-absorption studies, the oestrogen extracts were dissolved in methanol (3 ml.). In addition, the absorption spectra of the oestrogen extracts after the Kober reaction were determined. All the spectra were recorded on a Beckman DK-2 ratio-recording spectrophotometer.

RESULTS

Recovery of oestrogens added at different stages of the fractionation procedure

Table 1 shows that the purification procedures cause little or no significant loss of oestrogen. The recoveries of oestrogens after the Girard reaction and the Celite transfer are not shown in this table but have been described previously (Givner *et al.* 1960).

Specificity studies on each of the six urinary-oestrogen fractions

Late-pregnancy urine (500 ml.) was taken through the procedure as described in the Methods section and the six fractions were submitted to the different tests.

Retention volume. The retention volumes (V_r) of the six oestrogen fractions were in agreement with the values of pure standards (16α-hydroxyoestrone and 16-oxo-oestradiol-17β were used as reference standards for the ring D α-ketolic fraction). In some unpublished experiments, pure samples of the six oestrogens were added to male urine and the fractionation procedure was carried out. The Kober reaction was carried out on each of a large number of small eluate fractions from the Celite columns and the results showed no evidence that the characteristic retention volumes of the added oestrogens were affected by contaminants from the urinary extracts. Since the position of a compound in a chromatogram depends on its partition coefficient, the retention volume is of some value in its identification.

Paper chromatography of the six oestrogen fractions. The 2-methoxyoestrone, 16-*epi*oestriol and oestriol fractions showed single spots which had the same chromatographic mobilities as the pure reference compounds.

Oestrone fraction. Chromatography revealed three spots, two of which had mobilities identical with reference oestrone and 16-oxo-oestrone. When saponification was applied to the column eluate, the number of spots was reduced to two. Alkali washing (stage G, Methods section) proved superior, for chromatography after this procedure yielded a single spot with the mobility of oestrone and on elution gave a Kober reaction.

Ring D α-ketolic fraction. This fraction separated into a deep-blue spot, with mobility like that of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β standards, and a pale-blue spot between the 16-*epi*oestriol and 16-oxo-oestradiol-17β standards. These areas were eluted and subjected to further studies (see below).

Oestradiol-17β fraction. Chromatography revealed three spots. The spot with the mobility of oestradiol-17β gave a positive Kober reaction, whereas the two contaminants did not. No ketonic (oestrone) material was present in this fraction.

Kober-reaction spectra of the six oestrogen fractions. Kober reactions were carried out on pure compounds and on each of the six fractions and the absorption spectra recorded. The wavelengths of peak absorption of the fractions were in good

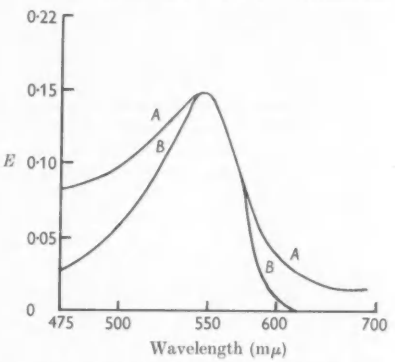

Fig. 2. Kober-reaction absorption spectrum of a 2-methoxyoestrone urinary fraction (*A*) with a modified reagent. *B*, 2-Methoxyoestrone (10 μg.).

Table 1. *Recovery of oestrogens added at different stages of fractionation procedure*

The recovery of oestrogens after the Girard reaction and the 'Celite transfer' have been reported elsewhere (Givner, Bauld & Vagi, 1960).

Oestrogen (10 μg.) added	No. of expts.	Stage investigated	Recovery (%) Mean	Recovery (%) Range
2-Methoxyoestrone	8	F*	96	91–100
Oestradiol-17β	11	M*	96	89–102
Oestriol	7	I	95	90–100
Oestrone	5	G	99	98–101

* The use of electric heaters (e.g. Electrothermal Bunsen) for the saponification stages gave higher recoveries of added oestrogen and decreased etching of glassware.

agreement with the appropriate reference standard (e.g. Fig. 2).

Ultraviolet-absorption spectra of the six oestrogen fractions. All fractions except 16-*epi*oestriol showed curves comparable with that of the reference compound (e.g. Fig. 3); 16-*epi*oestriol did not because of the presence of impurities.

Absorption and fluorescence spectra of the six oestrogen fractions in 75 % (v/v) sulphuric acid

Oestrone, 16-epioestriol and oestriol fractions. These fractions and the pure reference steroids showed maximum absorption and fluorescence at similar wavelengths (e.g. Fig. 4).

Ring D α-ketolic fraction. Fig. 5 illustrates that 16-oxo-oestradiol-17β fluoresces at a slightly shorter wavelength than does 16α-hydroxyoestrone. A solution containing 16-oxo-oestradiol-17β (10 μg.) and 16α-hydroxyoestrone (12 μg.) showed maximum fluorescence at a wavelength characteristic of 16α-hydroxyoestrone (Fig. 6). The '16α-hydroxyoestrone spot' showed maximum fluorescence at the wavelength associated with 16α-hydroxyoestrone (Fig. 6) or at an intermediate wavelength between the 16α-hydroxyoestrone and 16-oxo-oestradiol-17β peaks (Fig. 5). The unidentified 'x spot' showed maximum fluorescence at a wavelength which was slightly longer than the typical fluorescence peak of 16-oxo-oestradiol-17β (Fig. 5).

If it is assumed that the fluorescence emitted/unit wt. was the same for the '16α-hydroxyoestrone' and 'x spots', then approximately 10 % of the ring D α-ketolic fraction was made up of 'x'

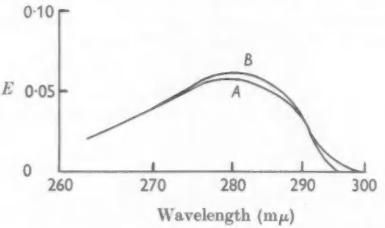

Fig. 3. Ultraviolet-absorption spectrum of an oestriol urinary fraction (*A*). *B*, Oestriol (50 μg.).

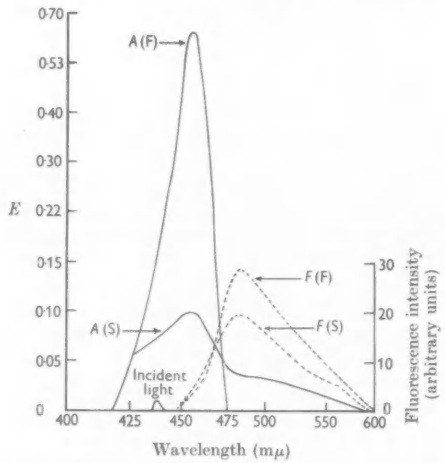

Fig. 4. Absorption (*A*) and fluorescence (*F*) spectra of 10 μg. of oestriol (S) and oestriol urinary fraction (F) in 75 % (v/v) H_2SO_4.

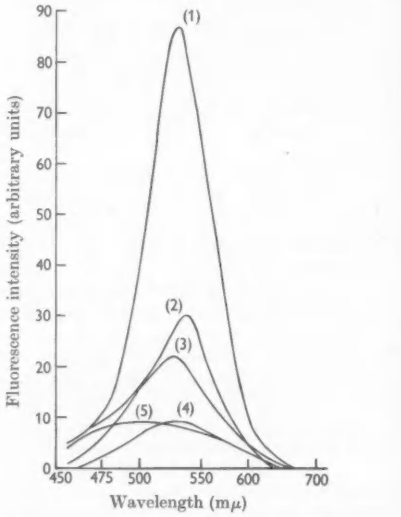

Fig. 5. Fluorescence spectra of (1) the eluted '16α-hydroxyoestrone spot' (from ketolic fraction), (2) 16α-hydroxyoestrone (12 μg.), (3) 16-oxo-oestradiol-17β (10 μg.), (4) eluted 'x spot' and (5) eluted paper blank in 75 % (v/v) H_2SO_4.

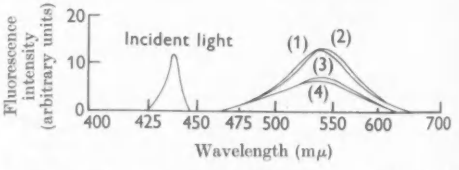

Fig. 6. Fluorescence spectra of (1) 16-oxo-oestradiol-17β (10 μg.), (2) 16-oxo-oestradiol-17β (10 μg.), plus 16α-hydroxyoestrone (12 μg.), (3) eluted '16α-hydroxyoestrone spot' (from ketolic fraction) and (4) 16α-hydroxyoestrone (12 μg.) in 75 % (v/v) H_2SO_4.

compound or compounds. Moreover, the corrected extinction of 'x spot' in the Kober reaction (with Oestriol reagent) was approximately 11% of the corrected extinction of the '16α-hydroxyoestrone spot' under similar conditions.

Fig. 7 illustrates that the absorption spectra of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β are different in aqueous sulphuric acid and that a solution containing the two oestrogens had an absorption spectrum characteristic of 16α-hydroxyoestrone. The '16α-hydroxyoestrone spot' had a wavelength of maximum extinction which was slightly shorter than the peak wavelength of 16α-hydroxyoestrone.

2-Methoxyoestrone and oestradiol-17β fractions. These fractions did not show well-defined spectra because of the presence of impurities.

Colour reactions on the six oestrogen fractions

'Blue tetrazolium' reaction. The method of Izzo, Keutman & Burton (1957) proved to be satisfactory when the ethanol was distilled from a 'blue tetrazolium' solution. Pure 16α-hydroxyoestrone, '16α-hydroxyoestrone spot' and 'x spot' gave positive reactions and were estimated by readings against an appropriate paper-blank solution. Tetrazolium reactions are given by compounds with an α-ketol or dihydroxyacetone side chain (Axelrod, 1955).

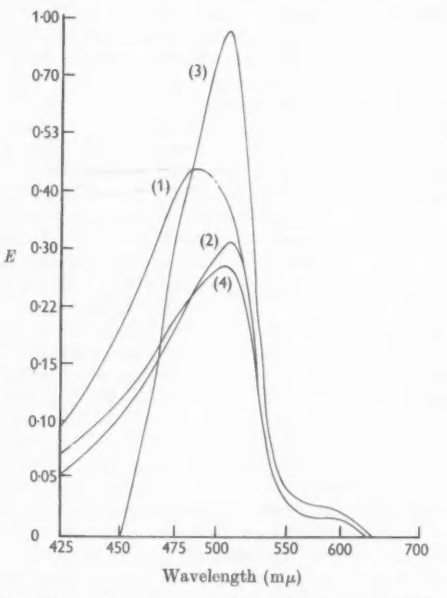

Fig. 7. Absorption spectra of (1) 16-oxo-oestradiol-17β (20 μg.), (2) 16α-hydroxyoestrone (24 μg.), (3) 16-oxo-oestradiol-17β (20 μg.) plus 16α-hydroxyoestrone (24 μg.) and (4) eluted '16α-hydroxyoestrone spot' (from ketolic fraction) in 75% (v/v) H_2SO_4.

Zimmermann reaction. The 2-methoxyoestrone, oestrone and ring D α-ketolic oestrogen fractions gave positive reactions by the procedure of Sobel, Golub, Henry, Jacobs & Basu (1958). This reaction is given by 17-oxo steroids (Klyne, 1957).

David reaction. The oestriol fraction gave a positive David (1934) reaction. The test was inconclusive with the 16-*epi*oestriol fraction because of the presence of impurities. The reaction appears to be specific for oestriol, 16-*epi*oestriol and their 3-methyl ethers; 17-*epi*oestriol, 16-oxo-oestradiol-17β- and 16α-hydroxyoestrone do not give the reaction (Diczfalusy, Tillinger & Westman, 1957).

Standard calibration curves prepared with pure 2-methoxyoestrone, 16α-hydroxyoestrone and 16-epi-oestriol. Since curves have not been reported for these oestrogens in the Kober reaction, it was considered essential to investigate this before recovery experiments were attempted. The results showed a satisfactory linear relationship between the concentrations and extinctions of 2-methoxyoestrone (0–10 μg.), 16α-hydroxyoestrone (0–12 μg.) and 16-*epi*oestriol (0–9 μg.) solutions.

Recoveries of oestrogens added to enzymically hydrolysed urine. Known amounts of the six pure oestrogens were added to portions of male urine and the oestrogen content of each fraction was determined after the fractionation procedure. At the same time urine blank values (in duplicate) were determined and used to correct the recovery experiment for endogenous oestrogen. Table 2 shows that the recovery was, on the average, from 65 to 95% depending on the oestrogen added. The lowest recovery was obtained with 16α-hydroxyoestrone.

Sources of error

The precautions reported by Bauld (1956) are of equal importance in this method. Heavy metals, dust, traces of cleaning solution, pitted glassware and solvent contamination are particularly destructive. In addition, care is required for the temperature control in the evaporation of methanol from the Celite pad containing the oestrogen extract.

Samples analyses

In preliminary experiments oestriol and the ring D α-ketolic oestrogens were measured in one-twentyfifth of a 24 hr. collection of male urine. In one subject, 28 μg. of oestriol and 40 μg. of ring D α-ketolic oestrogens per 24 hr. were excreted. These values were corrected for known experimental losses, which were determined by the recoveries of added oestrogen to a portion of the analysed urine. Recently, by the use of this

Table 2. *Recoveries of oestrogens added to normal male urine*

No. of urines analysed	No. of determinations	Oestrogen added (μg.)	Recovery (%) Mean	Recovery (%) Range
6	12	2-Methoxyoestrone (9·8)	95	92–97
6	12	Oestrone (5·1)	90	88–94
6	12	16α-Hydroxyoestrone (7·9)	65	57–71
6	12	Oestradiol-17β (5·0)	66	62–67
6	12	16-*epi*Oestriol (4·6)	87	83–92
6	12	Oestriol (5·0)	76	74–81

oestrogen method, Dr R. Hobkirk (personal communication) measured the 24 hr. urinary-oestrogen excretion of a normal pregnant woman (35 weeks) and found 315 μg. of 2-methoxyoestrone, 1890 μg. of oestrone and 4020 μg. of ring D α-ketolic oestrogen. In another pregnant woman (30 weeks), Dr Hobkirk found no 2-methoxyoestrone, 1775 μg. of oestrone, 3960 μg. of ring D α-ketolic oestrogen, 578 μg. of oestradiol-17β, 578 μg. of 16-*epi*oestriol and 12 620 μg. of oestriol in a 24 hr. urine sample.

DISCUSSION

The instability of 16-oxo-oestradiol-17β to hot-acid hydrolysis in urine (Watson & Marrian, 1955) and the known losses of oestrone, oestradiol-17β and oestriol during acid hydrolysis (Marrian, 1956) emphasized the need for a hydrolytic technique other than by acid treatment.

Katzman, Straw, Beuhler & Doisy (1954) found evidence that the major portion of oestrone, oestradiol-17β and oestriol is excreted in conjugation with glucuronic acid and that hydrolysis with bacterial β-glucuronidase resulted in urinary-oestrogen estimations equal to or higher than the values obtained by acid hydrolysis. Gallagher *et al.* (1954) reported that ox-liver β-glucuronidase is an effective procedure to hydrolyse urinary oestrogens. Brown (1957) found that in non-pregnancy urines, enzymic hydrolysis with a preparation of β-glucuronidase and phenol sulphatase from the limpet *Patella vulgata* resulted in higher values for urinary oestrone, oestradiol-17β and oestriol than those obtained with acid hydrolysis. Engel (1957) administered radioactive oestradiol-17β to human subjects and found that, for the most part, the urinary radioactive fraction was hydrolysed with 2 units of bacterial β-glucuronidase/ml. of urine on incubation for 2–48 hr. Recently, Dr R. Hobkirk (personal communication) found that Glusulase was more effective than bacterial β-glucuronidase in the hydrolysis of oestrogen metabolites in late-pregnancy urine. Thus in our method for urinary-oestrogen analysis, Glusulase was used for the hydrolysis of oestrogen metabolites in pregnancy urine and bacterial β-glucuronidase for male urine.

An important step in this chemical method to measure six urinary-oestrogen fractions included the use of the Girard reaction for the separation and purification of the ketonic oestrogens. Recent experiments on this reaction (Givner *et al.* 1960) indicated that under certain conditions a quantitative and non-destructive separation is obtainable.

For the specificity studies on the complete method, late-pregnancy urine (500 ml.) was used. The object here was to provide a searching test for the purification and separation procedures. The behaviour of the six oestrogen fractions on the partition columns, the paper chromatograms and in the multiple-extraction procedures supported the specificity of the method. The fluorescence and absorption spectra of the fractions in aqueous sulphuric acid, the ultraviolet spectra in methanol, the different colour reactions (Kober, Folin & Ciocalteu, 'blue tetrazolium', Zimmermann, David) and the Girard reaction provided additional evidence in support of the method's specificity. Recently, a 25-tube countercurrent distribution in the system benzene–water of a 16-*epi*oestriol fraction obtained from human-pregnancy urine (by the method described above) exhibited a single peak with the same K value as authentic 16-*epi*-oestriol (Dr R. Hobkirk, personal communication). In these latter experiments, [^{14}C]oestriol (2000–5000 counts/min.) added to pregnancy urine and carried through the method did not contaminate the 16-*epi*oestriol fraction to a significant extent; [^{14}C]oestrone (2000–4000 counts/min.) did not contaminate the oestradiol-17β fraction nor was it detectable in the first 10 ml. of eluate from the

non-ketonic columns; ^{14}C-labelled 16-oxo-oestradiol-17β (2000 counts/min.) did not contaminate the oestradiol-17β fraction. Thus the results indicated the satisfactory separation of urinary 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol.

On the basis of the fluorescence and absorption spectra of the eluted ketolic zones from the paper chromatograms, the ring D α-ketolic fraction was composed chiefly of 16α-hydroxyoestrone and 16-oxo-oestradiol-17β. An unidentified compound (or compounds) was also detected in this fraction. The properties of the unidentified compound were similar to those of 16α-hydroxyoestrone. First, the fluorescence spectra in aqueous sulphuric acid were alike. This property may be significant because of the different spectral characteristics of the ring D α-ketolic oestrogens when compared with the other oestrogens (Bauld *et al.* 1960). Secondly, both compounds gave the 'blue tetrazolium' reaction, suggesting that the unknown compound was an α-ketol. Marrian, Watson & Panattoni (1957) have reported that 16α-hydroxyoestrone has marked reducing power in the 'blue tetrazolium' reaction. Finally both compounds had similar spectra in the Kober reaction. However, the unidentified compound had lower mobility on a paper chromatogram than 16α-hydroxyoestrone (in this system 16α-hydroxyoestrone and 16-oxo-oestradiol-17β showed the same chromatographic mobility). Recently, Layne & Marrian (1958) isolated 16α-hydroxyoestrone, 16β-hydroxyoestrone and 16-oxo-oestradiol-17β from pregnancy urine. Thus the unidentified compound in the ring D α-ketolic fraction might have been 16β-hydroxyoestrone. It is improbable that the unidentified compound was 18-hydroxyoestrone (Loke, Marrian & Watson, 1959) since the fluorescence spectrum of the latter is unlikely to be similar to that of 16α-hydroxyoestrone.

It seems likely that the low concentration of 2-methoxyoestriol (Fishman & Gallagher, 1958) relative to oestriol did not permit its detection in the non-ketonic fraction during the specificity studies.

The accuracy of the method was estimated by the percentage recovery of six oestrogens added to hydrolysed male urine. The Kober reaction (Bauld, 1954) was used for the final determinations because of its established reliability and specificity. The recoveries were 65–95 % and the individual values were dependent on the nature of the added oestrogen. The relatively low recoveries of 16α-hydroxyoestrone (65 %) were to be expected on the basis of known losses in the Girard reaction and in the Celite-transfer technique. The recovery experiments were carried out on low concentrations of oestrogens in order to simulate the oestrogen content in male urine. Recently, Dr R. Hobkirk (personal communication) obtained the following mean recoveries by this method: oestrone (unlabelled and ^{14}C-labelled compounds added to non-pregnancy and pregnancy urines) $86 \pm 4{\cdot}6$ %; 16α-hydroxyoestrone (unlabelled compound added to non-pregnancy urine) and 16-oxo-oestradiol-17β (^{14}C-labelled compound added to pregnancy urine) $61 \pm 3{\cdot}9$ %; oestriol (labelled and unlabelled compounds added to non-pregnancy and pregnancy urines respectively) $80 \pm 4{\cdot}2$ %; oestradiol-17β (unlabelled compound added to non-pregnancy urine) $80 \pm 6{\cdot}6$ %. In no case, up to the present time, has there been any difference in the recovery between small amounts of unlabelled oestrogens added to non-pregnancy urine and ^{14}C-labelled oestrogens added to pregnancy urine.

A larger number of analyses are required before the precision can be evaluated adequately by statistics. The available experimental data indicate very good agreement between the duplicate estimations and therefore satisfactory reproducibility. Recently, Dr R. Hobkirk (personal communication) found that the precision of the newer method (Table 3) compared favourably with Bauld's method (Bauld, 1956). However, the following points should be noted: first, the values in Table 3 are for oestrogen analyses on pregnancy urine and not for recovery experiments; secondly, the procedure was shortened by the omission of the second Girard reaction and the refluxing with alkali procedure for the oestradiol-17β and 2-methoxyoestrone fractions. The omission of refluxing with alkali procedure is possible when the oestrogen to chromogen ratio is high as in late-pregnancy urine.

The sensitivity of the method has not been determined. An important limiting factor is the sensitivity of the Kober reaction. Under appropriate conditions, pure 2-methoxyoestrone (0·7 μg.), oestrone (0·3 μg.), 16α-hydroxyoestrone (0·6 μg.), oestradiol-17β (0·4 μg.), 16-*epi*oestriol (0·4 μg.) and oestriol (0·4 μg.) can be measured accurately. The experiments showed that six oestrogen fractions can be measured in as little as 20 ml. of late-pregnancy urine and that the ring D α-ketolic oestrogens and oestriol can be measured in 100 ml. of male urine. Greater sensitivity would be achieved by the use of fluorimetric methods, which remain to be applied to this new method.

The method is satisfactory for the determination of 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β, 16-*epi*oestriol and oestriol in pregnancy urine. This is equally true for male urine with the possible exception of the 2-methoxyoestrone fraction, where the high ratio of non-specific chromogens to oestrogens may create

Table 3. *Standard deviation between duplicates of consecutive analyses of oestrogens (μg./24 hr.) from pregnancy urines*

Data from Dr R. Hobkirk (personal communication).

Oestrogen	Method	Urinary oestrogen range (mg./24 hr.)	N*	s*
Oestrone	Bauld (1956)	0·3–1·0	206	0·043
	Bauld (1956)	1·0–3·0	76	0·067
	Givner *et al.*	0·4–2·4	44	0·074
Oestradiol-17β	Bauld (1956)	0·1–0·4	224	0·019
	Bauld (1956)	0·4–0·8	38	0·031
	Givner *et al.*	0·2–1·0	34	0·053
Oestriol	Bauld (1956)	1·0–10·0	157	0·295
	Bauld (1956)	10·0–30·0	149	0·607
	Givner *et al.*	3·0–23·0	30	0·510
16-*epi*Oestriol	Givner *et al.*	0·2–1·0	34	0·045
Ring D α-ketolic	Givner *et al.*	1·0–10·0	40	0·314
2-Methoxyoestrone	Givner *et al.*	Insufficient data	—	—

* $s = \sqrt{(\epsilon d^2/2N)}$, where s = estimate of standard deviation, d = difference between duplicates and N = number of duplicate analyses (Snedecor, 1952).

difficulties in the application of the Allen colour-correction formula. However, the presumed low concentration of 2-methoxyoestrone in male urine make this difficulty of doubtful importance.

In 8 working days one person can measure the oestrogen content of four different urines (in duplicate) and thereby measure 48 fractions. The method is easily modified for the measurement of only 2-methoxyoestrone, or oestrone or ketolic oestrogens.

SUMMARY

1. A new chemical method which separates and quantitatively measures 2-methoxyoestrone, oestrone, ring D α-ketolic oestrogens, oestradiol-17β,16-*epi*oestriol and oestriol in human urine is described.

2. The method involves: enzymic hydrolysis; ether extraction; separation of the ketonic from the non-ketonic oestrogens by the Girard reaction; purification of the non-ketonic fraction by refluxing with alkali and alkali washes; separation and purification of the oestradiol-17β, 16-*epi*-oestriol and oestriol fractions by column-partition chromatography; purification of the oestradiol-17β fraction by refluxing with alkali; separation and purification of the 2-methoxyoestrone, oestrone and ring D α-ketolic oestrogen fractions by column-partition chromatography; purification of the 2-methoxyoestrone fraction by refluxing with alkali and the oestrone fraction by alkali washes. The purified fractions are measured by the Kober-colour reaction with a spectrophotometric correction for non-oestrogen chromogenic substances.

3. The efficiency of the different procedures is described.

4. The method is evaluated for accuracy, specificity, adaptability and sources of error.

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